Measuring the effect of the tail-cuff protocol on central blood pressure in the mouse

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Measuring the Effect of the Tail-cuff Protocol on Central Blood Pressure in the Mouse

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Abstract

The mouse is commonly used in cardiovascular disease research. Therefore, accurate and reliable measurement of blood pressure in conscious mice is essential. Blood pressure can be measured directly from a major systemic blood vessel using implantable devices (telemetry probes) or non-invasively from a peripheral site such as the tail. Telemetry allows continuous collection of data in freely moving mice, whereas the mice are restrained during the tail-cuff procedure. However major surgery and higher cost associated with telemetry result in the fact that the tail-cuff technique remains widely used. The tail-cuff technique is known to cause stress in the animals and therefore its reliability and validity are questioned. The hypothesis is that the handling technique may reduce stress associated with the tail cuff technique. The aim of this thesis was to perform a detailed analysis of different handling techniques and the stages of the tail-cuff technique in their impact on central blood pressure, heart rate and core body temperature using telemetry.

I have revealed that all interventions caused significant increases in these parameters, with the restraint step inducing the largest change, which is maintained throughout the restraint period. These were not further exacerbated by increasing temperature or tail-cuff inflations. I saw no differences due to different handling techniques or the operator's gender.

Having compared the simultaneous recordings obtained by telemetry and the tail-cuff in the same mouse, I found that the tail-cuff system consistently produced lower readings than telemetry. However, the readings obtained by the tail-cuff were similar to those obtained by telemetry in non-disturbed mice on the same day in normal and hypertensive mice. The results show that the tail-cuff technique induces a stress response in mice, which is not alleviated by repeated exposure. Although these results support the use of the tail-cuff for monitoring blood pressure in the hypertension model, they also highlight the fact that measuring the blood pressure from the tail may not be representative of the systemic blood pressure. This is important new knowledge for researchers who use murine models of hypertension.

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Abbreviations

Abbreviation	Meaning
ACE	Angiotensin converting enzyme
Ang II	Angiotensin II
ASPA	Animal Scientific Procedures Act
AT	Applanation tonometry
bpm	Beats per minute
DBP	Diastolic Blood Pressure
ECG	Electrocardiogram
HR	Heart Rate
MAP	Mean Arterial Pressure
mmHg	Millimetres mercury
NTS	Nucleus tractus solitarii, nucleus of the solitary tract
PP	Pulse Pressure
RAAS	Renin Angiotensin Aldosterone System
RM-ANOVA	Repeated measures analysis of variance
SD	Standard Deviation
SEM	Standard Error of the Mean
SBP	Systolic Blood Pressure
VPR	Volume Pressure Recording

Chapter 1

Introduction

1.1. Cardiovascular system

The cardiovascular system is employed by all complex organisms for fast transport of oxygen, nutrients, waste products and heat. The organs of cardiovascular system are the heart, vasculature and the blood. Mammalian heart is a dual pump, connecting pulmonary and systemic circulations in series. The right ventricle of the heart receives the venous return and pumps it through the pulmonary arteries and capillaries for gas exchange. The left ventricle thereafter supplies the oxygenated blood to the systemic circulation, where all organs are connected in parallel. The vascular network delivers oxygen to approximately 15µm of most cells, i.e. within the optimal range for diffusion. Organs and tissues therefore rely on adequate access to flow that can be provided by healthy vasculature.

The geometry of the cardiovascular system itself can be understood in terms of the constructal theory that states that evolution favours structures that increase flow (Razavi et al., 2014). Interestingly, constructal theory applies to animate and inanimate structures to include plants, animals, river basins and social and economic structures (Bejan, 2005; Bejan and Lorente, 2010, 2013). In many social cultures the heart was regarded as the centre for body, the spirit and the soul (Barr, 2014). Also, the role of cardiovascular system in health and disease has been appreciated since antiquity.

Our modern understanding of the cardiovascular system is credited to William Harvey's publication of his famous work "De Motu Cordis" in 1628. In that book, he showed using experimental observation and deductive reasoning that blood circulates around the body propelled by the action of the heart (Fink, Hayes and Soni, 2008). Until then, Galen's view of a one-way system, propelled by the active dilation of the arteries, persisted in the western world for over 1500 years, in spite of the discovery of the pulmonary circulation by Ibn al Nafis in the middle 13^{th} century (Aird, 2011). Greek philosophers are regarded as the fathers of empirical research (Lloyd, 1979), which is the corner stone of today's science. Human dissection was allowed for some 30-40 years in Alexandria at the beginning of the Ptolemeic Dynasty period, i.e. at around 300 BC. It is around this time that Herophilus, an ancient Greek physician who practiced in Alexandria, is considered to be the first to perform systematic dissections of the human body. He made important discoveries with regards to the cardiovascular and nervous systems (Bay and Bay, 2010) and is reported to have come close to the discovery of circulation (Ghasemzadeh and

Zafari, 2011). Although ancient Indian physicians are believed to have an accurate understanding of cardiovascular system and circulation (Patwardhan, 2012), it took another nearly two millennia for the scientific method to develop, including human dissections and animal experimentation, to enable the discoveries, such as that made by William Harvey.

This work is largely concerned with the systemic circulation, and the words vasculature and circulation will refer to the systemic part of it unless specified otherwise.

1.2. Blood pressure

1.2.1. Basic concepts

Blood pressure is the pressure of circulating blood on the blood vessel walls and is measured relative to atmospheric pressure in millimetres of mercury (mmHg). A pressure gradient is required to propel the blood along the vessels (i.e. the flow). This gradient is created by ventricular ejection that raises aortic pressure to approximately 100 mmHg above the atmospheric pressure, whereas the pressure in the central veins is close to atmospheric pressure. There are four other factors that contribute to pressure within the vasculature: 1- hydrostatic (gravity), 2- elasticity of blood vessels (vessel compliance), 3- viscous resistance and 4- inertia of the blood vessels (that opposes blood velocity). Furthermore, blood volume and blood vessel length and diameter are other important factors that affect blood flow and pressure. The relative importance of each of these pressure components is different in different parts of the cardiovascular tree.

Blood pressure is most commonly understood in terms of the peak and trough values of the pressure wave: for example, 120/80 mmHg, where “120” corresponds to the systolic and “80” to the diastolic pressure. The units of blood pressure (mmHg) are associated with the earlier techniques to measure blood pressure. Introduced by Jean Leonard Marie Poiseuille in 1828, it made the technique more quantifiable and feasible which promoted its use, and thus the progress in the cardiovascular research (Booth, 1977; O’Brien and Fitzgerald, 1994; Naqvi and Blaufox, 1998). Until this day, physiologists express blood pressure (BP) as the height of the mercury column that is displaced by the pressure exerted by the intraluminal blood:

$$BP = p*g*h$$

Where p = liquid density, g = gravitational constant, h = height of the liquid of the liquid column.

Poiseuille dedicated most of his career to understanding flow of blood. To simplify the problem, however, he modelled this subject using simple liquids (such as water and alcohol) and narrow glass tubes. He published his experimental results in 1839 that Hagenbach used to derive the relationship of the factors that govern the rate of flow. The law is thus named Hagen-Poiseuille equation (Rampling, 2007):

$$Q = \Delta P \frac{\pi r^4}{8\eta L}$$

This can be rearranged for pressure:

$$\Delta P = Q \frac{8\eta L}{\pi r^4}$$

Where Q = flow, ΔP = pressure gradient along the vessel wall (in the direction of blood flow), r = vessel radius, η = blood viscosity, L = vessel length.

As per the conditions of Poiseuille's experiment, this relationship applies to laminar non-pulsatile flow in uniform non-distensible tubes, none of which are characteristic of the conditions of arterial flow. For example, it was shown that the flow is proportional to the third power of the radius when the flow is pulsatile. However a combination of the factors such as tapering and vessel branching in the right proportions can result in the system that is haemodynamically equivalent to the cylinder (Nichols et al., 2011). Other factors include the Windkessel effect of the larger conduit arteries (Belz, 1995) in combination with the varying distensibility profile of the arterial tree (Shadwick, 1999), plus the markedly higher resistance to flow in the arterioles evens out the pulsatile flow in the microcirculation. Therefore Hagen-Poiseuille law is commonly used to approximate the flow and pressure in arteries (Nichols et al., 2011).

Navier-Stokes equations are derivations of the Newton's second law of motion (F=ma) for fluids of constant mass and are used as the basis for blood flow modelling to include the parameters such as pulsatility, vessel elasticity and tapering. Hagen-Poiseuille's equation is in fact a solution of the Navier-Stokes equation for steady flow in rigid tubes and is commonly used as an approximation model. Considering the complexity of the arterial tree and the lack of information about certain physiological parameters, nearly all the available models are treated as approximations that are able to predict some but not other parameters depending on the input variables (Barnard et al., 1966). This makes physiological observation of paramount importance and indicates caution in extrapolating between different models.

For example, the model for pulsatile flow in non-tapering elastic vessels predicts attenuation of the pressure wave with distance and both pressure and velocity amplification in constricting taper, whereas physiological observation is the pressure wave amplification and velocity attenuation with distance (Barnard et al., 1966). Pulse wave travels at similar speeds in the human and mouse arterial trees; moreover, the human and mouse aortae have a number of similar ratios. However, the absolute central aorta length in the mouse is just under 50mm versus 50cm in humans. Empirical measurements showed there is no pulse pressure amplification in the mouse (Aslanidou et al., 2016).

The pressure of blood within the vessels is pulsatile in nature and is propagated through a vascular network of complex geometry and morphology. Although it is generally understood in terms of the peak and trough values in the clinic or otherwise approximated in terms of its mean value, pressure magnitude changes throughout the cardiac cycle and is different in different parts of the circulation. With the typical pressure of around 100 mmHg seen by the heart in the healthy circulation at rest, the pressure wave undergoes amplification and thereafter decays in the peripheral arteries and the microcirculation. The steepest pressure drop occurs in the arterioles and reaches approximately 30 mmHg at the pre-capillary level. Arterioles are the site of highest resistance; it is the combined resistance of microcirculation to flow that necessitates the high perfusion pressure that the heart needs to generate.

Non-linear viscoelastic properties of the blood vessels are very important factors that govern the blood flow and pressure propagation in the vascular network. The major blood vessels dilate passively during each systole and then recoil elastically to help the propagation of blood flow to the periphery. Each cardiac cycle generates a complex pressure wave, the shape (or the frequency components) of which is the product of the cardiac contraction and the varying viscoelastic properties of the vessel walls that it travels through. The combination of elastic and stiff fibres, mainly elastin and collagen respectively, are the structural components of the vessel walls that confer this property to the arteries. These components are functionally conserved and are the most important determinants of the flow and pressure characteristics in all species with closed circulatory systems. Even though fish and amphibians have pressures lower than the animal species, microstructural adaptations in the vessel walls ensure that the elastic modulus and thus the wave velocity are conserved. Typical wave velocity in the aortae in all species is 24 ms^{-1}. Elastin is a rubber-like polymer that has elastic modulus also comparable to rubber (1 MPa), whereas collagen is the strengthening component that is 1000 times stiffer than elastin. Elastin fibres offer initial resistance to flow at very low pressures and are most responsive to stretch in the range of physiological pressure with the steepest gradient between 70 and

90 mmHg in mammals. There is twice as much elastin as collagen in the aortic arch, and the ratio becomes reversed in the abdominal aorta (Shadwick, 1999). The ability of the arteries to expand and recoil has the pulse reducing and thus the flow smoothing effect downstream: flow wave amplitude to mean flow ratio in humans becomes three times less in the femoral artery compared to the aortic arch (Shadwick, 1999).

Changes in stiffness and bifurcations that take place along the vessel walls lead to wave reflections and the summation of the original and the reflected waves. This ultimately results in the amplification of the pressure wave from the centre to the periphery within the major arteries, such as the brachial. Pulse amplification results in the marked increase of the peak pressure and some reduction in the diastolic pressure, while mean pressure is relatively unaffected. For example, a pressure wave may have the amplitude of 30 mmHg, with the peak value typically around 100 mmHg, in the aortic arch; in optimally elastic arteries this pressure wave is amplified, with the peak pressure reaching 110 or 120 mmHg in the brachial artery (Nichols et al., 2011). Age-related stiffening of the elastic arteries that commonly happens in humans of over 65 years of age, results in faster wave propagation and pulse reflection. This leads to the in-phase summation of the forward and the reflected wave closer to the heart with the ultimate result that central and peripheral pressure wave amplitudes become more similar (Nichols et al., 2011). It has been shown that it is the pressure that the heart "sees", i.e. the pressure in the aorta rather than the pressure in the brachial artery, that is most predictive of the cardiovascular risk, and some drug therapies only affect the pressure in the peripheral arteries without the corresponding reduction in the aortic pressure (Williams et al., 2006).

Blood pressure is a complex wave that can yield valuable information about the condition of the cardiovascular system; it is a growing field of study and is a highly promising diagnostic and research tool that is becoming more widely used (Avolio et al., 2010).

The current clinical practice still largely involves the measurement of blood pressure from the brachial artery and to focus on the peak and trough values of the pressure wave, i.e. systolic and diastolic pressures. Diastolic pressure is understood to reflect the vascular tone, while systolic pressure depends on vascular compliance, cardiac filling and contractility (Sayk et al., 2015). Pulse pressure is the difference between systolic and diastolic pressures and mean pressure is the mean pressure over the cardiac cycle that can be defined as the area under the pressure curve divided by the cardiac period (Geddes, 2013). However it is often calculated as a sum of diastolic pressure and one third of pulse pressure based on the assumption that diastole occupies 2/3 and systole 1/3 of the cardiac cycle (Rogers and Oosthuyse, 2000). Systolic and diastolic pressure are the chief readouts of the cardiovascular system that currently guide the diagnosis and treatment.

In spite of the criticism of the current practice, use of these parameters has been a crucial tool in the past century that led to the dramatic improvement of management of hypertension (Kotchen, 2011). Systolic and diastolic pressures are one of the most important measured parameters in the cardiovascular research and the window used to assess the health of the cardiovascular system.

1.2.2. Homeostatic blood pressure regulation

Blood pressure is the mechanism of the cardiovascular system to redistribute flow to different organs to cope with various external and internal challenges and demands, such as supply of enough oxygen to the skeletal muscle during exercise, temperature regulation, etc. The main mechanisms to achieve changes in blood pressure are:

- Change in heart rate and/or stroke volume that directly affects the cardiac output
- Change in the diameter of blood vessels, commonly peripheral arterioles, that has direct impact on the resistance to flow
- Change in the blood volume, which is the more long-term mechanism that potentially affects both peripheral resistance and stroke volume.

Otherwise the relationship between all these variables can be demonstrated as follows:

Blood pressure = Cardiac Output x Total Peripheral Resistance

Cardiac Output = Heart Rate x Stroke Volume

Adequate blood pressure is necessary for appropriate organ perfusion and therefore it is one of the vital signs alongside with respiratory rate, oxygen saturation and temperature – the parameters regulated or otherwise closely linked to the circulatory system. When the blood pressure is below a certain threshold, perfusion of vital organs is compromised, which can be life-threatening (e.g. in septic shock). Abnormally high pressure in the arteries damages the inner lining of the vessels that may lead to plaque formation and consequent narrowing and stiffening of the vessels. High pressure may weaken the arteries and lead to aneurysms that may potentially rupture. The organs that are most susceptible to damage as a result of hypertension are the heart, brain, kidneys, eyes, and those that comprise the reproductive tract.

The most dangerous aspect of hypertension is that the symptoms are difficult to detect until the damage is done, therefore hypertension is called “the silent killer”. Thus, blood pressure is an

important indicator of cardiovascular health and risk for many adverse events such as stroke and heart disease. Therefore, blood pressure is probably the most measured clinical sign.

Homeostatic regulation of blood pressure is executed within a narrow range using the nervous system as the key mechanism. Blood vessels have intrinsic ability to regulate the flow via the endothelial and myogenic pathways. However the role of the nervous system is to integrate the series of inputs to rapidly coordinate the adequate perfusion of the vital organs such as the heart and the brain, and redistribute the flow to the other organs to meet the environmental and physiological demands (Thomas, 2011).

The involuntary control of all the organs and system of the body, including the cardiovascular, is carried out by the autonomic nervous system. The vasomotor centre is located in the medulla oblongata in the brainstem. It receives ascending impulses from the baroreceptors, chemoreceptors, mechanoreceptors, thermoreceptors located on the blood vessels, the heart, lungs, skin, skeletal muscle and visceral organs. It also receives the signals from the hypothalamus, cerebral cortex and other parts of the brain. Nucleus tractus solitarii (NTS) in the dorsomedial region of the medulla is the site of the first synapse of the ascending signals from major systemic arteries and cardiopulmonary area and the descending neurones. The projections from the NTS follow to the ventrolateral medulla (VLM) and the intermediolateral (IML) sites.

The sympathetic and parasympathetic are the branches of the autonomic nervous system that are responsible for the regulation of blood pressure and can be distinguished anatomically that is related to the distinct classes of effects these branches orchestrate. The sympathetic nervous system is associated with the “fight or flight” response and the parasympathetic nervous system is associated with the “vegetative” or resting state. Sympathetic activation can be approximated to increased heart rate and force of contraction, constriction of some vascular beds such as that associated with the gastrointestinal tract, increase in blood pressure and other effects that would be beneficial for a “fight or flight” response. Parasympathetic activation, on the contrary, slows down the spontaneous firing rate in the SA node in the heart thus reducing the heart rate and promotes other functions more associated with the resting state. Parasympathetic neurones innervate the heart and a small number of blood vessels, whereas sympathetic neurones innervate the heart, blood vessels, kidneys, including the adrenal glands. Therefore, the sympathetic system is the chief regulator of the cardiovascular function. The parasympathetic system, however, has a more limited effect, but important role on the heart.

Noradrenaline is released from the sympathetic postganglionic neurones and acetylcholine is the effector of the parasympathetic system. Both systems innervate the SA and AV nodes with

opposing effects on the chronotropy and dromotropy (conduction velocity). Sympathetic neurones activate the β1 adrenergic receptors in the heart that are Gs-coupled and thus increase cAMP concentration. This increases the rate of depolarisation in the SA node and the conduction velocity in the AV node and therefore the conduction rate and ultimately the heart rate. While in the myocytes β1 activation potentiates Ca^{2+} currents across the cells membrane and re-uptake in the SR, thereby increasing the force of contraction and the relaxation rate. Taken together, the result is the increase in the heart rate and the force of contraction.

Sympathetic neurones directly innervate most, if not all, arteries, arterioles and veins that have the smooth muscle layer. Noradrenaline is one of the main mediators released from the nerve terminals; it can mediate vasoconstriction or vasodilation depending on the receptor types involved. Vasoconstriction is mediated via the α1 or α2 types of adrenergic receptors located on the vascular smooth muscle. The α1 receptors are Gq-linked proteins that potentiate Ca^{2+} currents that initiate the following signalling cascade: Ca^{2+} activates calmodulin-dependent myosin light chain kinase that phosphorylates myosin light chains which are required for the myosin ATPase activation and the binding of the myosin and actin filaments to mediate the contraction of the vascular smooth muscle. The α2 receptors that are located on the smooth muscle inhibit the cAMP and also contribute to vasoconstriction. However the pre-synaptic α2 receptors inhibit noradrenaline release and thus reduce provide negative feedback to reduce the vasoconstriction (Thomas, 2011). Sympathetic vasodilation is mediated via the β adrenergic receptors, which is an important mechanism to enhance the blood flow to the tissues with the increased oxygen demand. The role of all three receptor subtypes has been identified in mammalian species and the role of β1 was shown to predominate that of the β2 in mouse and human vasculature (Chruscinski et al., 2001).

The baroreceptor reflex is the sympathoinhibitory reflex that is activated at each systole thus providing the beat-to-beat control over the blood pressure. A blood volume overload or an increase in blood pressure is sensed by low-pressure receptors located in the heart and great veins, and by high-pressure receptors in the carotid sinus, respectively. This information is relayed to the nucleus of the solitary tract (nucleus tractus solitarii, NTS) of the brainstem, where it is integrated. The NTS provides excitatory input to modulate both sympathetic and parasympathetic function. Stimulation of the NTS activates the caudal ventrolateral medulla, which provides inhibitory input for the rostroventrolateral medulla, where sympathetic tone is thought to be generated. At the same time, stimulation of the NTS activates the dorsal vagal nucleus of the vagus and nucleus ambiguus, where parasympathetic activity is generated. Thus, an increase in blood pressure leads to activation of arterial baroreceptors and activation of the

NTS, which induces parallel inhibition of sympathetic tone (through inhibition of the rostroventrolateral medulla), activation of parasympathetic tone (through activation of the dorsal vagal nucleus of the vagus). Sympathetic efferent fibres run through the IML columns of the spinal cord and make their first synapse in paravertebral autonomic ganglia, where postganglionic fibres originate to innervate the vasculature and the heart. Vagal fibres run through the vagus nerve and synapse in ganglia located within target organs. Thus, the initial increase in BP ultimately leads to inhibition of sympathetic tone to the vasculature (generally resulting in vasodilation) and to the heart (resulting in a decrease in cardiac output), and activation of parasympathetic tone to the heart (leading to a decrease in heart rate). These actions restore BP to baseline values. Thus, the baroreflex provides continuous and instantaneous regulation of BP (Thomas, 2011).

1.2.3. The renin angiotensin aldosterone system (RAAS)

The renin angiotensin aldosterone system (RAAS) is a key regulatory mechanism of blood pressure that comprises these factors.

Renin acts on pro-angiotensin and then angiotensin 1 is cleaved to angiotensin II by angiotensin converting enzyme (ACE). This enzyme is found on vascular endothelial cells and because of the large area of the lung endothelium, this site is thought to be the major site of conversion. Kidneys are the key sensors of blood pressure and renin is released into the blood stream. It sets of the chain of events to produce angiotensin II (Ang II). Ang II has a range of pro-hypertensive effects, including in the brain. It acts as an acute vascular constrictor agent, it stimulates thirst receptors in the brain. It also has less acute effects, it is used as a widely used rodent model to induced hypertension. This leads to secondary changes in the heart where the workload of the heart is increased, leading to cardiovascular adverse remodelling including fibrosis. During this process there is a resetting of the baroreceptor to work at a higher set point. It has a proven role in humans as ACE inhibitors and Ang II receptor antagonists are both effective in humans to lower blood pressure, thus indicating the importance of the rodent model.

Other mediators involved in the regulation of blood pressure include nitric oxide, CGRP, bradykinin, histamine, prostaglandins and endothelin as shown in figure 1.1.

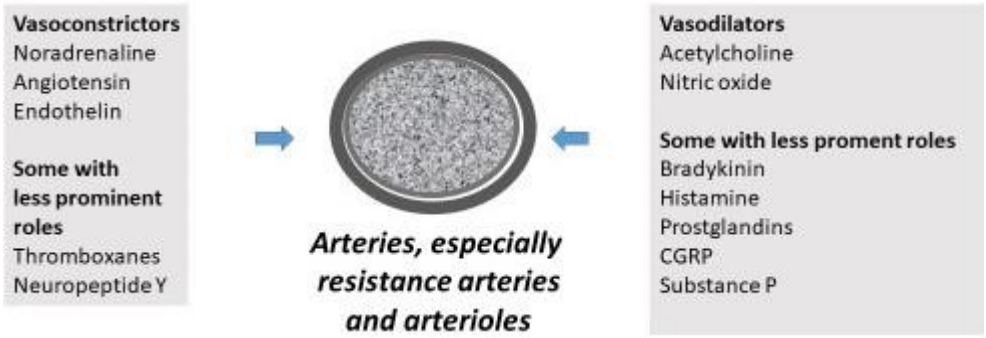


Figure 1.1. Established mediators that are known vasodilator/constrictors. *Those that are vasoactive, but with a less established role in the mechanisms involved in the control of blood pressure are also listed. (Adapted from an original by S Brain, unpublished)*

To conclude, there are numerous systemic and local regulatory mechanisms in place to ensure that blood flow to certain organs and tissue meets the demand dictated by internal and external factors. In terms of research it is essential to measure BP in laboratory animals.

1.3. Hypertension

It has been known for over a century that chronically raised blood pressure is associated with morbidity and mortality. The lability of blood pressure as a consequence of emotional stress and activity has been long recognised (Osler, 1912), and it appears to have complicated the interpretation of raised blood pressure as a clinical problem. What is the "normal" range of blood pressure range has been a matter of debate since the discovery of the ways to measure it. The accepted "normal" values have changed over the years as higher blood pressure was increasingly recognised as a risk factor for cardiovascular diseases. At the beginning of 20th century many physicians shared the belief that systolic blood pressure above 160mmHg may be the necessary adjustment to maintain adequate blood flow to the organs, especially in association with advanced age (Osler, 1912); however the idea that the normal systolic pressure is 100 plus age was questioned by early 1930s. (Hay, 1931)

While the physicians remained sceptical regarding the value of obtaining the blood pressure readings as late as the 1930s (Hay, 1931) and hypertension as a pathology was not recognised well into the 1940s (Moser, 1986); the Insurance Industry recognised the diagnostic value of sphygmomanometer as early as 1906 and started building evidence of the dangers associated with raised blood pressure. As a result, life insurance policies can be refused to individuals with pressure above 150/105 mmHg (Kotchen, 2011).

The concept of hypertension now is different from how it was perceived some seventy years ago (Timmermann, 2006) and it keeps evolving. The current definition of "optimal" blood pressure is

below 120/80 mmHg, which is a notch down from the previously accepted140/90mmHg, of which the range 130 – 139mmHg systolic and 80-89mmHg diastolic pressure are defined as "stage 1 hypertension (Whelton et al., 2017; Carey et al., 2018).

Table 1.1. Classification of blood pressure in adults according to the 2017 High Blood Pressure Clinical Practice Guideline (Whelton et al., 2017).

Category	Average* blood pressure, mmHg		
	Systolic		Diastolic
Normal	<120	and	<80
Elevated	120-129	and	<80
Stage 1 hypertension	130-139	or	80-89
Stage 2 hypertension	≥ 140	or	≥90

*Average blood pressure is based on the average of ≥2 careful readings obtained on ≥ 2 occasions according to the recommendations laid out in section 4 of the guideline (Whelton et al., 2017).

A blood pressure below 90/60mmHg can seriously undermine organ perfusion and is a fatal complication of septic shock (as mentioned above), as well as generally a concern for patient management under surgery. In general practice, it is the hypertension, especially the systolic part (Lindenstrøm et al., 1995; Kannel, 2000; Sesso et al., 2000) that is the major risk factor for cardiovascular and diseases and morbidity (Whelton et al., 2017). Owing to the progress made in understanding blood pressure and its regulation, together with, the availability of pharmacological tools, hypertension is classed as a modifiable, yet it was defined as the leading risk factor for disease burden worldwide for the period between 1990 and 2010 (Lim et al., 2012). According to World Health Organisation, in the UK cardiovascular diseases are responsible for 31% of deaths for population between the ages of 30 and 70 and hypertension is the risk factor, which affects 30.7% of men and 27.0% of women (World Health Organization – Non-communicable Diseases Country Profiles, 2014).

Blood pressure tends to increase with age and obesity (Kotchen, 2011), and women are relatively protected until the onset of menopause (Reckelhoff, 2001; Syme et al., 2009). As the tools to measure the blood pressure and its various parameters have been developed, our knowledge of the cardiovascular system has grown. Despite the great advances made, the fundamental question what causes the so called "essential hypertension" remains.

1.4. History of blood pressure measurement

The concept of blood pressure came into being with the first recorded measurement of blood pressure by Reverend Stephen Hales in 1711. It came to clinical practice by the end of the 19th century and the value of the blood pressure measurements became appreciated, albeit, with

some divide in opinions, at the beginning of the 20th century (Kotchen, 2011). Until then, pulse assessment had been an important diagnostic method in use since antiquity (Naqvi and Blaufox, 1998; Ghasemzadeh and Zafari, 2011; Saklayen and Deshpande, 2016). It allowed accurate diagnosis despite the apparent misconceptions about its aetiology. Pulse assessment remains as the centre point of medical examination in the traditional medicine such as Ayurvedic system and traditional East Asian medicine (Naqvi and Blaufox, 1998; Wang and Cheng, 2005; O'Brien et al., 2013; Bilton and Zaslawski, 2016).

1.4.1. The pulse

Pulse is a rhythmical throbbing of the arteries as blood is propelled through them (https://en.oxforddictionaries.com/definition/pulse, accessed on 20/03/2018). Ancient physicians in China, India, Egypt, Greece, and the Romans developed extensive guidelines on how to examine pulse and also made important observations regarding certain pulse types and links to diseases and prognoses that certain pulse types were thought to be associated with. Pulse examination largely involved qualitative observations that nevertheless offered remarkably accurate insights, that can now be quantitatively confirmed and one can appreciate the significance of those early observations (Naqvi and Blaufox, 1998).

In ancient Egypt, as well as in most other cultures, the heart was regarded as the centre of the body and the spirit and features prominently in the medical literature of the time. It was the only organ that was not removed during the mummification (Saba et al., 2006). Ancient Egyptians offer probably the earliest records of medical knowledge in the series of papyri dating between 1700 and 3000 BC (Barr, 2014). The best known are Ebers and Edwin Smiths papyri that are believed to be written between 1700 – 1500 BC. The Ebers papyrus is the main source of knowledge on cardiovascular pathology and Edwin Smith papyrus is the surgical textbook of the ancient Egypt. Both papyri were the compendia of medical knowledge gathered over the preceding centuries and based on earlier documents going back to around 3000 BC. It was then already recognised that the pulse originated from the heart and heart examination via pulse assessment was routinely made (Ghasemzadeh and Zafari, 2011; Barr, 2014). The Ebers papyrus contains references to diagnosing the conditions that are now understood as ventricular fibrillation, angina, heart failure, including cardiac dysfunction and congestive heart failure, through pulse palpation. It was observed that the heart weakens as a result of "the heart not speaking or the vessels not listening" (Saba et al., 2006). Although clear reference to counting the pulse is made in the Edwin Smith papyrus, this is not deemed as the true heart rate determination due to no mention of the time-keeping device (Saba et al., 2006; Barr, 2014). Interestingly,

however, the first device to measure pulse rate was built in Alexandria and used by a Greek physician Herophilius at around 300 BC (Saba et al., 2006; Ghasemzadeh and Zafari, 2011).

Pulse examination is presently one of the most important diagnostic tools in the traditional

Chinese and Ayurvedic medicine (Kaptchuk, 1983; Kurande et al., 2012) both going back nearly 5000 years in history.

The beginning of the Traditional Chinese Medicine is attributed to the Yellow Emperor, who is thought to have lived between 2697-2597 BC (Naqvi and Blaufox, 1998). The historians are not entirely sure if the Yellow Emperor was a real or a legendary figure. The work that is attributed to him was translated in 600 BC and may have been compiled by a number of people over the centuries (Naqvi and Blaufox, 1998; Ghasemzadeh and Zafari, 2011). The Yellow Emperor's Classic on Internal Medicine, Nei Ching, contains many references to the pulse and attempts to interpret the pathology in terms of its respective types. It probably offers the first description of hypertension as a result of excess salt in the diet and increased incidence of strokes in the affected individuals (O'Brien and Fitzgerald, 1994; Naqvi and Blaufox, 1998). Pulse characteristics were compared to sounds and actions such as "blows of the hammer", "like the notes of a string instrument" or "fish gliding through the water"; a "small and fine" pulse was considered a sign of a "painful" heart. There were a number of other later works, the most notable being the one by Wang She-He, also referred to as Wang Chu-Ho, entitled Maijing (Pulse Classic). This is a ten-volume compilation of all the knowledge to that date on the pulse examination and interpretation. The author describes four main characteristics of pulse: slow, rapid, superficial and deep, which are further subdivided into 24 types of pulse (Naqvi and Blaufox, 1998; Shu-he, 2007).

Ayurveda is an ancient system of medical knowledge that originated some five thousand years ago in the Indian subcontinent (Mishra et al., 2001). It understood the body in terms of three humours that represented the combination of the five earth elements (Horine, 1941). The pulse examination in both ayurvedic and traditional Chinese medical examination was performed by placing three fingers along, most commonly, the radial artery. The pulse sensations under each finger are to represent each body humour, the abnormality in each of those are used to interpret the cause of the disease (Kanad, 1891; Kaptchuk, 1983; Ghasemzadeh and Zafari, 2011). The ancient Indian philosopher and physician Sage Kanada, who is thought to have lived at around 600 BC (Ghasemzadeh and Zafari, 2011), wrote an important book for the system of the ayurvedic knowledge named Science of Sphygmica. The book offers detailed description of the method of how to examine the pulse, which anatomical locations can be used to diagnose various

conditions, time of the day when the assessments should or should be made, and so on (Kanad, 1891). A method for counting the pulse rate is also described and the differences in normal pulse rates for different age groups were also described (Ghasemzadeh and Zafari, 2011).

Although there is clear reference to pulse measurement in the ancient Egyptian and Indian literature, the first measurement of pulse rate is most commonly attributed to the Greek Physician Herophilius (335-280 BC) (Ghasemzadeh and Zafari, 2011), who is considered most likely adapted the Egyptian water clock to measure the patients' pulse (Saba et al., 2006). What is more, he built up empirical evidence of what a normal pulse rate should be in each age group and he compared the patient's pulse.

Ancient Greek philosophers started using experimental method and animal dissection and thus considered as the founders of our modern science. They also understood the health and disease as a balance of the humours, of which they distinguished four. Interestingly, the origin of this concept is probably based on the phenomenon of blood separation that was well known to the ancients (Rampling, 2007). Ancient Greeks built on the knowledge gathered by the Egyptians. Hippocrates (375BC) described pulse in several health conditions, however it was Praxagoras of Kos (340 BC) who was credited in the Greek literature as the first to use the pulse in medical examinations. He also discovered that pulsation only occurs in the arteries, not in the veins. His student Herophilius (335-280BC) continued his work on the pulse and he measured the pulse rate in his medical examinations, for which he built Clepsydra, the water clock. He recognised the pulse rate differences in different ages and genders. Erasistratus (304-250BC) recognised that pulsation in the arteries follows the contraction of myocardium, however at that time, it was believed that arteries contained air. It was several centuries later that Galen (129-200 AD) discovered that arteries actually carry blood (Ghasemzadeh and Zafari, 2011). He then went onto describe pulse based on its magnitude (i.e. the size of each arterial dilatation), frequency, speed of alternating systole ad diastole, and regularity (Wallis, 2000). Based on the combination of these variables, each pulse beat was described in terms of twenty seven characteristics (Ghasemzadeh and Zafari, 2011).

Medieval medicine also understood health as a balance of the body humours; the balance of these humours could be perceived via pulse assessment. Ibn Sina, also known as Avicenna (981-1037 AD) defined eight parameters to describe the pulse, such as: size of dilation (or the beat), its duration, duration of the pause after the beat, temperature of the pulse, artery compressibility, artery fullness/ emptiness, regularity of force and rhythm of the consecutive beats. Thus, Avicenna appears to be the first to describe resistance and elasticity of the arteries. He described pulses that are linked to weakened myocardium, atrial and ventricular arrhythmias.

His work was furthered by Moses Maimonides (1135 – 1204) who diagnosed various arrhythmias based on the findings from the pulse and also linked pulse irregularities with the severity of the conditions (Ghasemzadeh and Zafari, 2011).

Since the time of Herophilius in 300 BC, the next development in the measurement of pulse rate followed the discovery of the pendulum by Galileo and specific adaptation of the pendulum by Santorio Sanctorius in 1603 to count pulse rate. Interestingly, the unit for the pulse rate was inches (Moore, 1897). Counting of the pulse rate as we know it today was introduced by John Floyes nearly a century later, who used a minute clock for this purpose (Ghasemzadeh and Zafari, 2011).

The description of the greater circulation by William Harvey in 1628 is defined as the start of the modern era of Western medicine (Treacher, 2008). This discovery was made possible through persistent *in-vivo* experimentation in different species (Ghasemzadeh and Zafari, 2011), for which the revival and evolution of the scientific method over the few preceding centuries paved the way. Animal dissection was re-introduced in Salerno in the 12th century and human dissection in the late 13th century in University of Bologna. Although the objective was to demonstrate the teachings of Galen, this allowed to gradually identify and thus challenge the errors in the earlier ideas. The development of the quantitative argument and mathematics are also important factors that made Harvey's discovery possible and accepted by the community (Aird, 2011).

1.4.2. Rev Stephen Hales and blood pressure measurement

Although the concept of systole and diastole was described in Harvey's work, the concept of blood pressure measurement came into being with the experiments of Reverend Stephen Hales somewhere between 1707 and 1711. The development of the techniques to measure blood pressure in humans started with the measurements in animals, and in fact the first blood pressure measurement was performed in the horse (Hales, 1733; Booth, 1977). He measured blood pressure in horses (n=3), dogs (n=20) one sheep and one doe (deer) (Hales, 1733) and his work was published in 1733. The measurement of blood pressure was a terminal procedure and he used sick animals who were going to be put down (Hales, 1733). Reverend Hales' experiments on living creatures, that included dogs, horses, frogs etc, sparked opposition from the movement against "thoughtless cruelty to animals" that started at around that time and was headed by the famous poet Alexander Pope, who was a neighbour of Reverend Hales (Harre, 1983).

Hale's first experiment on the horse is also the most famous (Booth, 1977; Naqvi and Blaufox, 1998). He inserted a glass tube into a neck artery of a tied down conscious mare and observed a rise of blood in the tube to a height of 8 feet 3 inches high, which is equivalent to about 174 mm

of mercury (mmHg). He noted the oscillations in the column corresponding the heart beats and breathing. He went on to measure the pressure in the jugular vein. In the other 2 horses the blood pressure was measured as 214 mmHg in the carotid artery and approximately 20 mmHg in the jugular vein. The pressure reading for the dogs ranged between 60 and 150 mmHg for carotid artery and 2 and 16 mmHg for jugular vein. The variations were probably as the readings were taken as the animal was exsanguinated (Hales, 1733).

Hales also performed a series of experiments to measure the capacity of the left ventricle in an ox and several dogs. He calculated the blood velocity in the dogs' arteries based on the measured ventricular volume, aortic dimensions, arterial pressure and pulse rate. Estimating that the blood is propelled in approximately 1/3 of the time between the pulses, he arrived at the value of 1.62miles per hour (2.6km/h or 0.73m/sec), which closely compares to more recent measurements in 5 subjects that ranged between 0.46 and 0.67 m/sec (Segadal and Matre, 1987). Using man ventricular volume estimates an aortic measurements by Dr Keill (Hales, 1733), Hales estimated for the arterial pressure in man, which was 159 mmHg (Hales, 1733).

Figure 1.2. Hales' experiments to determine blood pressure of a horse by Cuzzort. *Courtesy of The Wellcome Collection. https://wellcomecollection.org/works/km4x3rye*

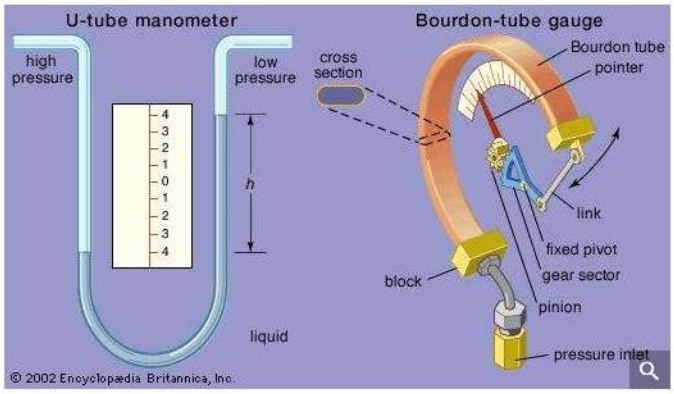
1.4.3. Poiselle and the mercury manometer

Using blood itself as a pressure indicator was inadequate on many accounts: it resulted in infections, thus precluding its use in human subjects or experimental animals other than terminal procedures and clotting of blood (Smith and Bickley, 1964). The mercury manometer developed by Poiseuille 100 years later addressed the shortcomings of the Hales' set-up to allow more accurate and widespread study of blood pressure. Jean Leonard Marie Poiseuille (17971869) developed the mercury manometer as part of his doctorate thesis in 1828. The device was called "haemodynameter" that consisted of a U-shaped tube filled with mercury that acted as a gauge for measuring pressure. The mercury manometer was connected to a cannula filled with potassium carbonate to act as an anticoagulant. The cannula was inserted in blood vessels (down to 2 mm size) of dogs and some other animals (Booth, 1977). Poiseuille dramatically refined the technique for measuring blood pressure by addressing the issue of blood coagulation and

infections. Using mercury as a pressure indicator also reduced the size of the column that made the apparatus easier to use. Thanks to Poiseuille's innovation, blood pressure measurement gained wider use and the units for blood pressure are millimetres of mercury (mmHg) to this date.

The other important development in the field of cardiovascular research and readily taken up by other branches of physiology was made in 1847 by Carl Friedrich Wilhelm Ludwig, a professor of physiology in Leipzig. He further developed Poiseuille's device by adding a float to the mercury column to which a stylus (originally consisting of a quill, a feather pen) could be attached. This enabled the production of traces on a rotating smoked drum. The instrument, called a. kymograph (from Greek "wave writer"), allowed visualisation and recording of consecutive pressure waves. The major significance of this invention is that it allowed the simultaneous objective recording of blood pressure (or rather relative changes of such), heart rate and respiratory frequency, which enabled the identification and study of the previously unrecognised physiological relationships (Bruce Fye and Willis Hurst, 1991). This principle of graphical recording was a major step and was readily taken up by other physiologists to record other processes such as muscle contraction and laid the foundation to many pharmacological assays that are in use today (Booth, 1977; Bruce Fye and Willis Hurst, 1991; O'Brien and Fitzgerald, 1994).

Due to the high density of mercury and consequently high inertia, mercury manometer systems have inadequate frequency responses for accurate recording of the pulse waveform and measurement of its peak and trough values, i.e. systolic and diastolic pressures in humans and animals. Efforts continued to find an alternative system with higher frequency response to reflect the pressure gradients generated by the action of the heart. Ludwig's student, Adolf Fick (1864) adapted the Bourdon tube (a flattened tube that straightens slightly under pressure) for intra-arterial recording of blood pressure, leaving the mercury column out of the design. This device for the first time allowed to record systolic and diastolic pressures relatively accurately, however it did not allow the detection of the dicrotic notch (Vlachopoulos et al., 2011). The inertia of the lever in Fick's device was recognised by Otto Frank (1903). Frank's device consisted of a pressure sensing membrane and a small mirror that could reflect the light following membrane deformation due to the pressure (Kahn, 1924; Geddes, 2002). High fidelity arterial waveform could be reproduced with this device for the first time (Vlachopoulos et al., 2011).



Two types of pressure gauge(Left) A U-tube manometer, in which differential pressure is measured as the difference *h* between the high-pressure reading and the low-pressure reading, multiplied by the density of the liquid in the tube. (Right) A Bourdon-tube gauge, in which a coiled tube, flattened into the cross section shown and attached to a fixed block, is open to a pressurized fluid. The tube straightens slightly under pressure to a degree measured by a pointer.

Encyclopædia Britannica, Inc.

Figure 1.3. Two pressure gauges. *Source: https://www.britannica.com/technology/pressure-gauge*

1.4.4. First blood pressure measurements in human

In 1856, French surgeon Jean Faivre used Ludwig's apparatus to record arterial blood pressures in humans during amputation surgeries. Commonly using femoral artery, he also attempted blood measurement in the aorta. He reported pressures of approximately 120mmHg in the peripheral arteries and estimated pressure in the aortae as 102 mmHg and consistent between individuals. Although it is unlikely that his recordings reflected the systolic pressures, he is credited with the first accurate blood pressure measurement in man (O'Brien and Fitzgerald, 1994; Naqvi and Blaufox, 1998).

Accurate direct measurements of systolic and diastolic pressures were nevertheless also made using a mercury manometer. Golz and Gaule (1878) engineered two oppositely directed check valves and selector valve into a mercury manometer: one of the valves only permitted the forward flow of blood and thus, following several heart beats, allowed the detection of the systolic pressure; the other valve only permitted backflow of blood and thus the diastolic pressure was measured (Geddes, 2002) which could be applied for invasive blood pressure measurements.

1.4.5. Development of non-invasive techniques

The development of non-invasive techniques to measure blood pressure was essential to make this tool useful for the clinic. The first non-invasive techniques more pertained to the study of pulse. The first such method was described in 1834 by a French physiologist Jules Herrison. The instrument consisted of a mercury reservoir and protruding graduated glass column. The reservoir was compressed against the radial artery until the pulsation would stop, at which point the systolic pressure could be estimated (O'Brien and Fitzgerald, 1994). The instrument was

however used for pulse assessment, rather than blood pressure (Herisson, 1835). Similar can be said about the later devices until probably Mahomed's work at the Guy's Hospital in the 1870s.

In 1855, Karl Vierodt, a professor of physiology at Tübingen, described an instrument that he named "Sphygmograph" (*sphygmos,* meaning pulse). He designed this apparatus to allow the non-invasive measurement of the pulse amplitude. The apparatus consisted of a cup with two sets of sensitive levers. The levers replaced the mercury column that he considered was inadequate to reflect the oscillations of the pulse wave due to under-damping (Mach, 2016). The cup was placed over the throbbing radial artery, and increasing weights were placed into the cup to obliterate the pulse. Arterial pulsations were reproduced on the smoked drum by the stylus connected to the cup via one of the sets of the levers. The apparatus was used in humans, however the cumbersome design and inadequate accuracy and sensitivity precluded its wide use (Booth, 1977; Naqvi and Blaufox, 1998). The apparatus failed to detect dicrotic notch. However the important achievement was to adapt the recording mechanism developed by Ludwig for invasive procedures to non-invasive application (Cameron and Hicks, 1996).

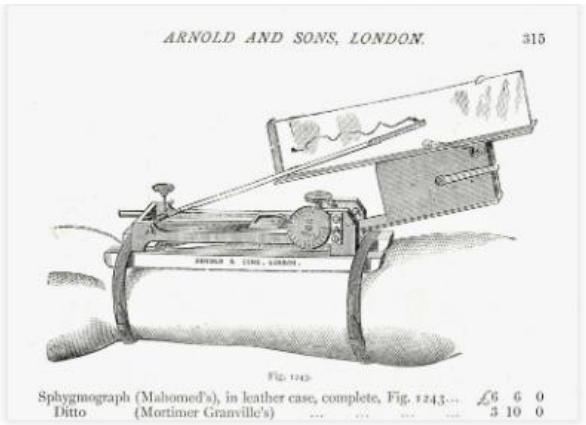
The clumsy design of Vierodt's invention inspired a French doctor, Etienne Jules Marey, to tackle the problem in 1860. The resulting instrument, a wearable sphygmograph, was the first to make its way into the clinic in Europe, Britain and America. Pressure in the arm was obliterated by placing the arm in the glass chamber that was filled with water; the column of water could be varied as needed. The water chamber was connected to the mercury manometer. The sphygmograph was strapped to the forearm, so that the pulsations of the radial artery were transmitted to the smoked paper that was moved by a clockwork mechanism. The pressure in the manometer that coincided with the maximum and cessation of pulse recording by the sphygmograph was recorded as estimates of arterial blood pressure. In spite of substantial improvement to the device, it was still unwieldy and it was only the kymograph part of the device that gained wide-spread acceptance in the clinical practice; thus the device was mainly used to study pulse (Booth, 1977; Naqvi and Blaufox, 1998).

Marey's other interesting modification to his sphygmograph was the transmission device, in which movement of the stylus was transmitted through a column of air in a tube, thus allowing recoding on a paper distant from the sphygmograph (Naqvi and Blaufox, 1998).]

The very first formal clinical study on pulse was done using this device by the inventor himself that describes age-related pulse differences (Ghasemzadeh and Zafari, 2011). Marey was also the first to point out the significance of the pressure point at which maximal amplitude of pulsations

was recorded. He however suggested that this corresponded to mean arterial pressure, not diastolic as later established (Naqvi and Blaufox, 1998).

John Burdon Sanderson is credited to introduce the sphygmograph into England; the first record of its use in English medical practice goes back to 1865 and is credited to Dr Francis Anstie (Foster, 1866). Sanderson, alongside with Foster, introduced more accurate graduation of the pressure on the radial artery. However this was to improve the quality of the pulse traces, not arterial pressure measurement (Cameron and Hicks, 1996). Frederick Akbar Mahomed, a physician at Guy's Hospital, built on Sanderson's work: among other modifications, he added a screw that acted as a pressure dial to accurately estimate the pressure required to occlude the artery. Interestingly, the pressure was measured in ounces (Cameron and Hicks, 1996; Naqvi and Blaufox, 1998). Mahomed is thus credited with the description of the first quantitative sphygmograph (Vlachopoulos et al., 2011) and he used his device (see figure 1.3) to describe the condition what we now refer to as "essential hypertension" (Cameron and Hicks, 1996; Naqvi and Blaufox, 1998).



Mahomed's sphygmograph from Arnold and Sons 1895 Catalogue of Surgical Instruments and Appliances (University of Edinburgh library). The device was strapped t the wrist and increasing pressure was exerted on the radial artery by adding weights until pulsations ceased. Sphygmographs continued in use into the 20th century.

Figure 1.4. Mahomed's sphygmograph. *Source: http://historyofnephrology.blogspot.com/2014/06/blood-pressure-is-linked-to-kidney.html*

Further British contribution to the field came from Robert Ellis Dudgeon, a physician who practiced homeopathy in London. He introduced a compact and fairly robust sphygmograph in 1880, involving a single wrist strap, which greatly promoted the popularity of the device in clinical practice and it persisted until 1920s (Cameron and Hicks, 1996; Naqvi and Blaufox, 1998). French cardiologist Pierre C.E. Potain realised that the spring in the manometer that was used for the attachment of weights to occlude the artery, interfered with the pressure recordings: he observed that the pressure needed to move the spring depended on both blood pressure and the resistance of the arterial wall, interfering with the readings (Booth, 1977).

In 1881, Karl Ritter von Basch, introduced further improvement, an inflatable rubber bag to occlude pulse in the radial artery. The bag was filled with water and placed over the radial artery. It included a manometer bulb filled with mercury and a hollow graduated glass column to register the rise of the mercury in the column and thus the pressure in the bag. Apparatus validated by simultaneous recordings from carotid artery in dogs and is the first apparatus to study haemodynamic pathology: it was shown that atherosclerosis is associated with hypertension and fever with hypotension. Further contribution to the apparatus by Potain was to replace the water with air in the pressure bag in 1889 (Booth, 1977).

The sphygmograph was accepted into clinical use by 1880s, yet it was initially criticised for "pauperising the senses" by the British Medical Journal. The objections were based on the use of numbers to provide a measurement, rather than the physician's personal senses as a result of physical examination (Booth, 1977; Brophy, 2009; Oparil, 2016). The techniques to record pulse and blood pressure were undergoing constant development and this is not an exhaustive list of these developments or the inventors who introduced them. Already by the end of the 19th century, attempts were also made to measure other parameters of the cardiovascular system. Such as that, C. Ludwig's student, Von Frey, made certain modifications to the sphygmograph that allowed him to estimate arterial elasticity. He referred to it as "tonograph" in his monograph on the study of pulse in 1892. He also refers there to the auscultation of the pulse before it was described by N.S. Korotkov in 1905 (Naqvi and Blaufox, 1998).

Scipione Riva-Rocci in 1896 introduced the method on which the current non-invasive technique is based upon. Brachial artery is occluded with an inflatable rubber cuff (ca 5 cm wide, enclosed underneath non-expandable material) around the whole circumference of the arm. He meticulously tested the device on animals and human cadaveric arm with artificial circulation (Booth, 1977). Palpation was used to detect whether the pulsation was present following the occlusion of the brachial artery, which did not allow determination of diastolic pressure. In 1897 Hill and Barnard introduced the design for manometer indicator needle sensitive to arterial pulsations: as the cuff deflated, the appearance of throbbing needle oscillations indicated systolic pressure, then the transition to larger magnitude and back to smaller needle oscillations indicated the diastolic pressure (Hill and Barnard, 1897). The cuff originally introduced by RivaRocci created acute angle edges between the outer edges of the cuff and the skin that artificially raised the pressure on the site – this was realised and corrected by Recklinghausen in 1901 and thus the cuff width was increased to 13 cm (Booth, 1977).The non-invasive techniques described so far can be classed as 'oscillometric' since they rely on the arterial pulsations for blood pressure estimation.

1.4.5.1. Korotkoff sounds

In 1905, a Russian army surgeon, N.S. Korotkoff (alternatively spelt as Korotkov), described the tapping sounds that can be heard when a stethoscope is placed over a brachial artery at the cubital fossa as the Riva-Rocci occlusion cuff is deflated. This introduced the auscultatory method to determine blood pressure, that was readily taken up into the clinic and further popularised by the binaural stethoscope, or phonendoscope (Booth, 1977).

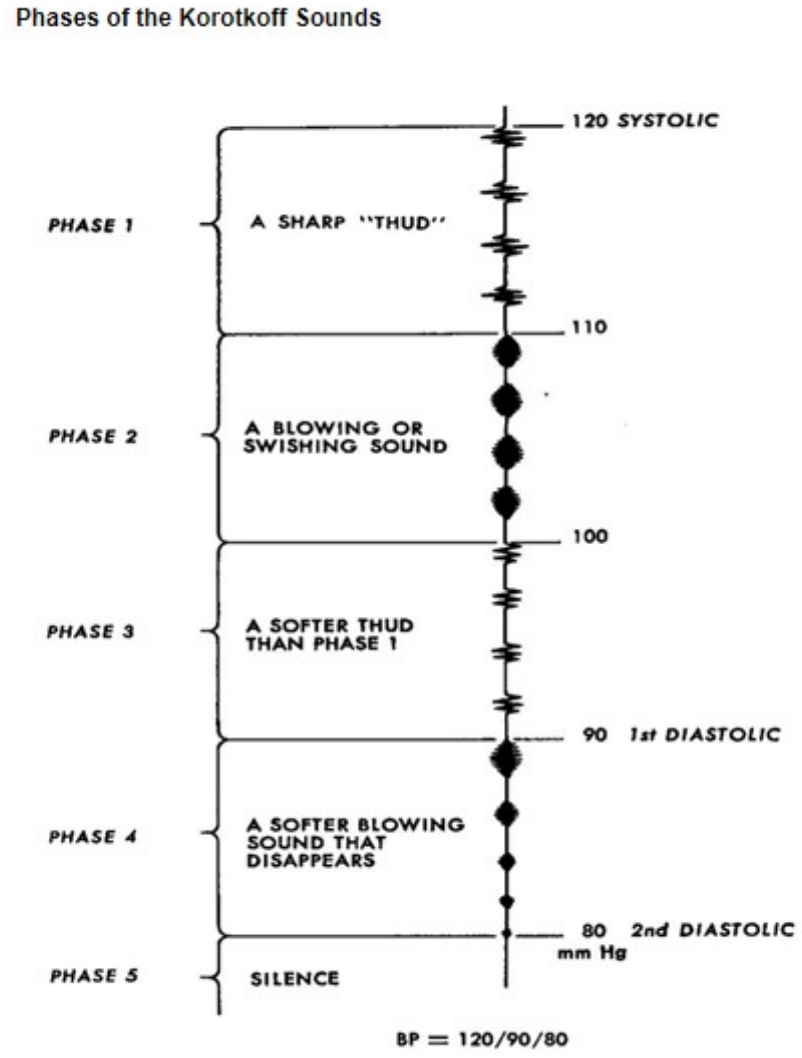


Figure 1.5. Correlation of the Korotkov sounds with blood pressure measurements. Source: WA Baum and co website. http://www.wabaum.com/faq.aspx#.XM_6UY5KjIU

Shortly after the original description of the arterial sounds by Korotkov, Ettlinger in 1907 described the 5 distinct phases as the pressure in the arterial cuff was released:

- phase I: appearance of the sound with a 'snapping' characteristic;
- phase II: continuous persisting murmurs;
- phase III: increasing of sound intensity above that of phase II;

- phase IV: muffling of sounds;
- phase V: cessation of sounds.

The Korotkov sounds arise due to the re-introduction of flow, which is turbulent, into the artery following the occlusion and the pulsation of the artery. This is a complex interaction between artery and the blood flow that can complicate the estimation of blood pressure using this method. Although determination of the systolic pressure involves registration of the cuff pressure at which the sound first appears and is relatively straightforward, the presence of "ausculatory gap" between phases I and II in certain patients, can complicate the matter (Avolio et al., 2010). With regards to the diastolic pressure, Ettinger originally suggested to use the fifth sound as a point for diastolic pressure. In 1908 J. Fisher argued for the phase four sound, when the sound changed abruptly to a dull or weak sound (Naqvi and Blaufox, 1998). The current recommendations are to use the fifth phase; however, if sounds persist, the fourth phase should be used (Avolio et al., 2010).

The oscillometric technique continued to be developed alongside the ausculatory. Innovation by Erlanger (1904) included registering the arterial pulsations by the upper arm cuff that was later used by Pachon (1909) in combination with an aneroid gauge (O'Brien and Fitzgerald, 1994). Although the mercury manometers and ausculatory method for blood pressure measurement have been the standard in the clinic for nearly 100 years, digital sphygmomanometers have now largely replaced mercury ones in clinical practice. The blood pressure measurement now largely relies on the oscillometric technique, i.e. measurement of the oscillations of the artery by the pressure sensors rather than auscultation for Korotkoff's sounds downstream at cubital fossa (Pickering et al., 2005; Myers, 2014).

Both methods use occlusion and subsequent reperfusion of the artery as the basic principle. The oscillometric method relies on the analysis of the oscillogram, i.e. the appearance, increase in amplitude and subsequent disappearance of the arterial oscillations during the deflation of the occlusion cuff. The point of the appearance of the oscillations is identified as the systolic pressure. Determination of the diastolic pressure is less straightforward as it is not always associated with the disappearance of the oscillations and many manufacturers use proprietary algorithms to determine the diastolic pressure (Avolio et al., 2010).

The earlier techniques, i.e those developed by Herrison, Ludwig, Vierodt, Marey, Mohamed, were aimed at recording pulse and gave a qualitative illustration of the pulse wave rather than the measure of force exerted by the blood. The actual blood pressure measurement was enabled

with the introduction of the brachial cuff by Riva-Rocci and description of the aortic sounds by Korotkov (Avolio et al., 2010). Since then, the pressure measurements were carried out in terms of the peak (systolic) and trough (diastolic) values of the arterial waveform taken from the brachial artery. Epidemiologic and prognostic value of these measurements have been established (Kotchen, 2011). It was assumed that the brachial pressure is representative of the pressure in the central aorta, however it is now understood that the vascular network has complex physiology (London and Pannier, 2010) and peripheral and central parts of the circulation can respond differently to certain pharmacological interventions and it is the pressure in the central arteries that carries the best prognostic value in cardiovascular risk assessment (Safar and Jankowski, 2009; Avolio et al., 2010; Nelson et al., 2010) .

The brachial cuff technique is criticised for the lack of accuracy and inadequate prediction of the central pressure (Avolio et al., 2010; Nelson et al., 2010). Therefore the clinical practice of the 20th century to focus only on the peak and trough values of the brachial waveform, thus disregarding the information that the pulse waveform can yield, is criticised by the experts (O'Rourke and Seward, 2006; Avolio et al., 2010; Hametner and Wassertheurer, 2017). Works of O'Rourke and many other scientists has revived the enquiry into this parameter that is coming to the limelight of cardiovascular research (Avolio et al., 2010; Chowienczyk, 2011). Applantation tonometry (AT) is a technique that allows reconstruction of the central arterial waveform noninvasively (Nelson et al., 2010) O'Rourke and his colleagues are credited with laying the foundation for this technique (Bartels et al., 2016), however its basic principle was described by Dr Maklakoff in 1885 to measure intraocular pressure (Stuckey, 2004). AT estimates central blood pressure by partially flattening a peripheral artery using a hand-held device that employs strain-gauge pressure sensor. The pulse waveform is decomposed into constituent waves and analysed using fast Fourier transformation (O'Rourke and Seward, 2006; Nelson et al., 2010). This century therefore sees the revival of the pulse analysis that started millennia ago, subjecting it to the quantitative analysis afforded by the current technology (Naqvi and Blaufox, 1998).

The devices and the methods for blood pressure measurement are now standardised and comprehensive guidelines exist for the best practice to measure blood pressure and hypertension diagnosis. Blood pressure measurements have extended beyond the clinic and the current recommendation is to supplement the office blood pressure measurement with the ambulatory blood pressure monitoring (ABPM) over 24 hours (Whelton et al., 2017).

Most of the underlying principles for measuring blood pressure or recording the pulse were developed in the 19th – early 20th century. The table below lists certain milestones in the development of the technique to measure blood pressure.

Table 1.2. Timeline of the development of blood pressure measurement

Year	Inventor	Description and notes
1733	Revd. Stephen Hale	First to measure blood pressure (in the horse).
1828	J.L.M. Poiseuille	Developed a U-shaped mercury manometer
1834	J. Herrison	Sphygmometer – first non-invasive device
1847	C. Ludwig	Kymograph – first intra-arterial pulse recording
1855	K. Vierodt	Sphygmograph – first non-invasive method to record pulse
1856	Faivre	First accurate measurement of intra-arterial blood pressure in man
1860	E.J. Marey	Substantial improvement of Vierodt's sphygmograph
1864	Adolf Fick	Attached lever to the hollow C-spring (Bourdon tube) adapted for blood pressure recording
1863	John Burdon Sanderson	More accurate graduation of Marey's sphygmograph
1865	Francis Anstie	The first record of sphygmograph use in English medical practice
1872	Frederique Akhbar Mahomed	Introduces a more precise mechanism to vary pressure on the radial artery and uses this to describe essential hypertension
1878	Golz and Gaule	Engineered a mercury manometer with oppositely directed check valves and selector valve that could allow to detect systolic or diastolic pressures (Geddes, 2002)
1880	R.E. Dudgeon	Improves the design of the sphygmograph that promotes its wide adoption in the clinical practice
1880	P.C.E. Potain	Realises the interference of the spring in the manometer with the accuracy of the recordings
1880	Karl Ritter von Basch	Inflatable rubber bag was used as a pressure to occlude pulse in the radial artery.
1885	Dr Maklakoff	Describes the theory behind the method of applanation tonometry as applicable to the measurement of intraocular pressure
1889	P.C.E. Potain	Water in the pressure bag is replaced with air.
1892	A. Mosso	Tonometer to measure arteriolar pressure (finger) (Kolls, 1920)
1892	M. von Frey	"Tonograph" to estimate arterial elasticity
1895	A. Mosso	Continuous blood pressure recording in man
1896	Scipione Riva-Rocci	Brachial artery is occluded with an inflatable rubber cuff (ca 5 cm wide)
1901	Recklinghausen	The cuff width was increased to 13 cm.
1897	Hill and Barnard	Introduce an arm cuff (independent of Riva-Rocci) and an aneroid manometer (Hill and Barnard, 1897)
1903	Otto Frank	Optical manometer (Frank capsule)

Year	Inventor	Description and notes
1905	Korotkoff N.S.	Auscultatory method: placement of stethoscope over cubital fossa downstream of the constricted brachial artery produced distinct sounds
1907	Ettinger W.	Described 5 distinct phases of the sounds originally described by N.S. Korotkoff
1909	M.V. Pachon	Introduces sphygmo-oscillometer, an aneroid device (O'Brien and Fitzgerald, 1994)
1924	(Kahn, 1924)	Frank capsule method is adapted to measure blood pressure in man
…		
1942	Wagner R.	Mechanical system to register blood pressure at radial artery in man using the principle of vascular unloading technique
1942	(Lilly, 1942)	The Electrical Capacitance Diaphragm Manometer
1945	(Grundfest et al., 1945)	Resistance wire strain gauge for intra-arterial blood pressure measurement and peripheral pulse recording in humans and animals
1959	Laurence	Catheter-tip manometer
1973	Peňáz J.	Vascular unloading technique on the finger (human) by means of an electro-pneumatic control loop.

This is by no way a complete overview to justly recognise all the contributors. Some inventors worked independently of each other arriving at similar results (such as Riva-Rocci and Barnard and Hill), in which case their contribution is usually recognised based on chronology. Many advances happened as a result of the progress made in the field of physiology or in other disciplines, such as materials science, mathematics or physics. I have largely focused on the adaptation of the relevant technology or method as applicable to cardiovascular measurements.

1.4.6. Developing techniques for the measurement of blood pressure in laboratory species

The development of the scientific knowledge, introduction of new materials and technology in the 20th century has led to the exponential growth of biomedical research. The techniques to measure blood pressure originally were developed using animals (Hales, 1733; Booth, 1977). Now the research industry relies heavily on experimental models, many of which are animal models. The focus of the part of the introduction that covers most of the 20th century will be the development of the techniques to measure blood pressure in the experimental animals.

The mouse is the most commonly used laboratory species today. Commercial breeding of mice for scientific research started in Massachusetts, USA in 1909 and C57BL6, one of the very first inbred strains, was established by 1920 (Hedrich, 2004). However certain qualities of mice as a

research species were recognised even before that time. Gregor Mendel started his famous experiment on inheritance using mice back in 1854 before he was forced to switch to peas (Henig, 2017). William Harvey is reported to have used mice in his work on circulation (Hedrich, 2004). The first set of experiments to measure blood pressure, however, was carried out in larger animals: horses, dogs, sheep and deer (Hales, 1732), as previously discussed. The benefit of using smaller animals in cardiovascular research was recognised in the early decades of the 20th century to replace cats and dogs that were more commonly used at that time. Therefore the techniques to measure blood pressure in smaller animals needed to be developed and characterised (Durant, 1927). The even smaller size of the mouse must have been a significant limiting factor for earlier blood pressure recordings, therefore most of the efforts to develop the devices for blood pressure measurement were aimed at rats. Even then, one of the earlier published studies on the blood pressure in the rats by Baldwin et al. in 1924 reported difficulties accessing and obtaining recordings from carotid from femoral or carotid arteries. The abdominal aorta was used instead (1924).

Shortly after this, Durant (1927) states the preference for cannulating the carotid artery due to less trauma to the animal compared to the abdominal aorta or iliac artery. In this study, a standard Luer needle (G20) is used as a cannula connected to the mercury manometer. The blood pressure in the rat is estimated around 119mmHg and interestingly the females are said to have lower blood pressure than males. The rat circulation was found to respond to pharmacological and electrical stimulation similar to humans. The mercury manometers are understood to have inadequate frequency response to study arterial waveform and the Frank capsule is indicated for this purpose.

Hamilton, Brewer and Brotman (Hamilton et al., 1934) report measuring pressure contours in intact small animals (mice, rats and some small birds) using a Frank capsule, a metal diaphragm manometer with a lens mirror. A hypodermic needle was inserted directly in an artery of a lightly anaesthetised animal. The apparatus has high enough frequency for accurate pulse contour capture. (Hamilton et al., 1934; Woodbury and Hamilton, 1937) The apparatus was later adapted to study the relationship between intrathoracic, intra-spinal and arterial pressures in humans (Hamilton, 1936).

1.4.7. Indirect measurement of blood pressure

The drive to develop non-invasive techniques to measure blood pressure in animals was answered the following year by Griffith (1934), who adapted the technique earlier developed for humans (Griffith and Collins, 1933) and Bonsmann (1934). Griffith developed the technique of

observing capillaries in the paws of anaesthetised rats following the release of the pressure cuff. Bonsmann reports the use of the photocell apparatus to record blood pressure from the tails of conscious rats and mice. Blood pressures in the range of 70 – 90 mmHg and 80- 100 mmHg respectively, were obtained. These values were criticised to be too low by Woodbury and Hamilton (1937). Diaz and Levy (1939) characterised the technique to estimate both systolic and diastolic pressures in anaesthetised rat by bleeding the tail. The change of the bleeding patterns as the pressure in the tail-cuff was released was used to estimate systolic and diastolic pressures.

Shortly before that, Byrom and Wilson (1938) in London developed a water plethysmograph to measure systolic blood pressure in the rat. The plethysmograph comprised a glass tube that terminated in a circa 0.5mm diameter graduated capillary. The plethysmograph was filled with warm water coloured with a dye to aid the detection of the water level in the capillary that corresponded to the volume changes in the tail. Because of the movement artefacts, the animals had to be anaesthetised.

The following year, Williams, Harrison and Grollman (1939) in the US developed the tail-cuff plethysmography for use in conscious rats and mice (a smaller version of the apparatus was built for mice). The apparatus included a holder for the animal, occlusion and plethysmograph cuffs. This plethysmograph was different from the one described above (Byrom and Wilson, 1938) in that it included the same kind rubber tubing as the occlusion cuff, but it held water that was in turn connected to the water manometer. As the blood started returning to the tail following the release of the occlusion cuff to the level of the systolic pressure, the water level in the manometer began to rise.

Vasodilation was the most important factor for the measurements using this technique. Animals had to be pre-heated to 40°C before the experiment and the restraint holder contained a heating element should repeated measurement take place. It was recommended that the temperature was maintained at around 37°C during the recording sessions. Inadequate heating was recognised as the most common source of error, as well as “inadequate training” of the animals. Overheating was said to cause restlessness and thus artificially high blood pressure readings; under-heating and more extreme overheating are reported to result in false low readings. Preliminary training to habituate the animals to the procedure was recommended. The authors acknowledge that the plethysmograph developed by Byrom and Wilsom was more sensitive, however it required anaesthesia.

This method developed by Williams et al. appears to use the same principle as the Volume Pressure Recording system (Kent Scientific) that is commonly used by researchers today.

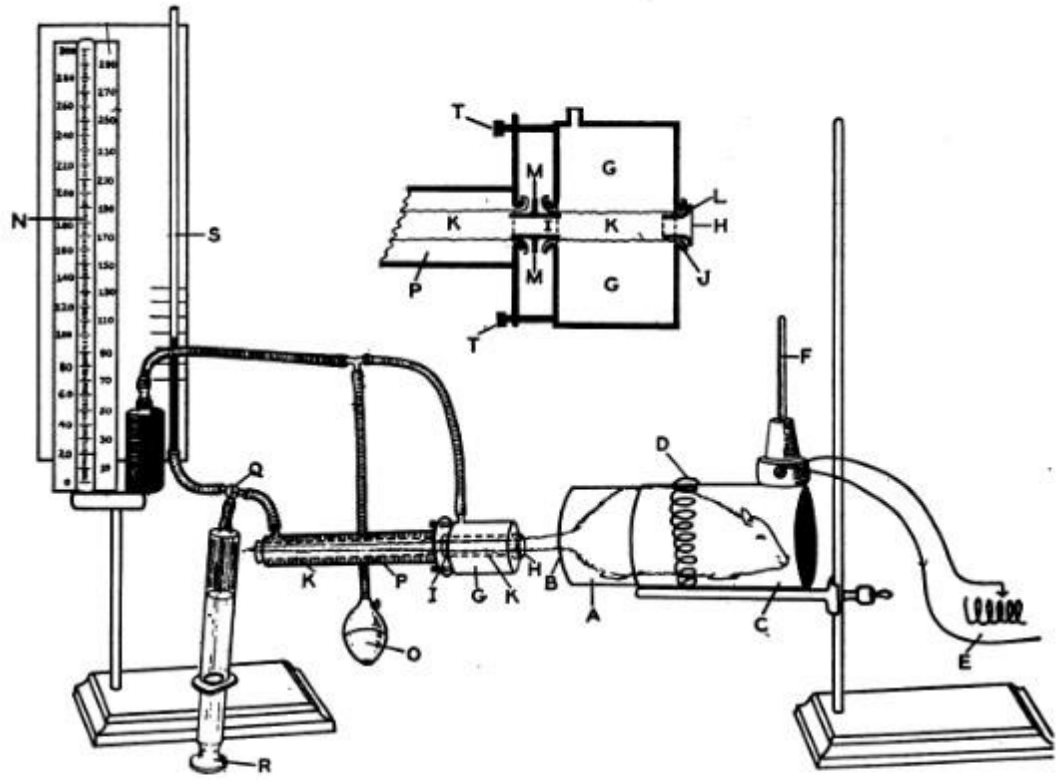


FIG. 1. BLOOD PRESSURE APPARATUS AND RAT HOLDER

A. Brass can for holding rat; *B*. Hole for rat's tail; *C*. Copper sleeve into which *A* slides; *D*. Heating unit from flat iron; *E*. Variable resistance; *F*. Thermometer; *G*. Pressure cuff; *H*. and *I*. Holes through which tail is drawn; *J*. Flange to hold rubber tubing; *K*. Thin-walled rubber tubing; *L*. and *M*. Metal insert to hold tubing between pressure and plethysmograph chambers; *N*. Mercury manometer; *O*. Inflation bulb and air release valve; *P*. Plethysmograph chamber; *Q*. Three-way tap; *R*. 20 cc. syringe; *S*. Water manometer; *T*. Screws for holding together the plethysmograph and pressure chambers.

The insert shows details of construction of blood pressure cuff and plethysmograph. For further details and operation, see text.

Figure 1.6. One of the earliest illustrations of tail-cuff system and the first prototype of the modern VPR system. Source: Williams et al., J. Clin. Invest. 1939, where they show their technique for measuring blood pressure in a rodent.

Other methods for indirect pressure measurement were tested that included tissue transillumination. McMaster modified Griffith's method that was described for rats in 1934 to anaesthetised mice (McMaster, 1941). Nail-bed capillary vessels of the mouse's hind paw were observed under the microscope following the occlusion and release of the blood flow to the paw. The method was validated against readings taken from the carotid using mercury manometer. Good agreement was found between the techniques, however the cuff pressure was approximately 5 mmHg below the carotid pressure. Algire (1949) described a membrane manometer system to measure blood pressure within the vasculature of the dorsal skin. A transparent chamber that can be pressurised to occlude blood flow, being connected to a mercury sphygmomanometer, was surgically inserted between the layers of the dorsal skin in the mouse. Conscious, but restrained animals could be observed under the microscope for blood pressure measuring using this device. This technique cannot be called non-invasive and implanted

chamber appeared to compromise animal's welfare; the technique was not further developed. Friedman and Freed (1949) described the use of a microphone to detect arterial sounds in the rat tail to measure systolic blood pressure, however this technique was not adopted by the research community either.

The non-invasive techniques currently available for mice predominantly uses the tail as a site for the measurements. The basic principle is to occlude the blood flow to the tail and then, following the gradual release of the pressure, to detect the reappearance of the pulse or measure the volume changes in the tail as the blood returns to the tail. Pulse detection technologies include photoplethysmography (uses a light source) and piezoplethysmography (uses piezoelectric crystals). A photoplethysmographic method was for rats and mice was described nearly 90 years ago (Bonsmann, 1934) and undergone further development to be used in humans (Weinman et al., 1960) and mice (van Nimwegen et al., 1973). Originally a photocell could only be used in anaesthetised albino mice or rats, however the later technologies can be applied in conscious pigmented animals (Koutnikova et al., 2009; Kratz et al., 2016). There are various sensor technologies available to detect blood flow in the tail that uses Doppler (Reddy et al., 2003) following reperfusion. Volume Pressure Recording (VPR) is said to be the relatively new sensor technology compared to photoplethysmography or piezoplethysmography, however the technique is similar to that developed by Williams et al (1939). It of the most commonly used sensor technologies that employs a differential pressure transducer to sense the volume changes (Malkoff, 2005) validated against the direct pressure measurements (Feng et al., 2008).

1.4.8. The direct measurement of blood pressure

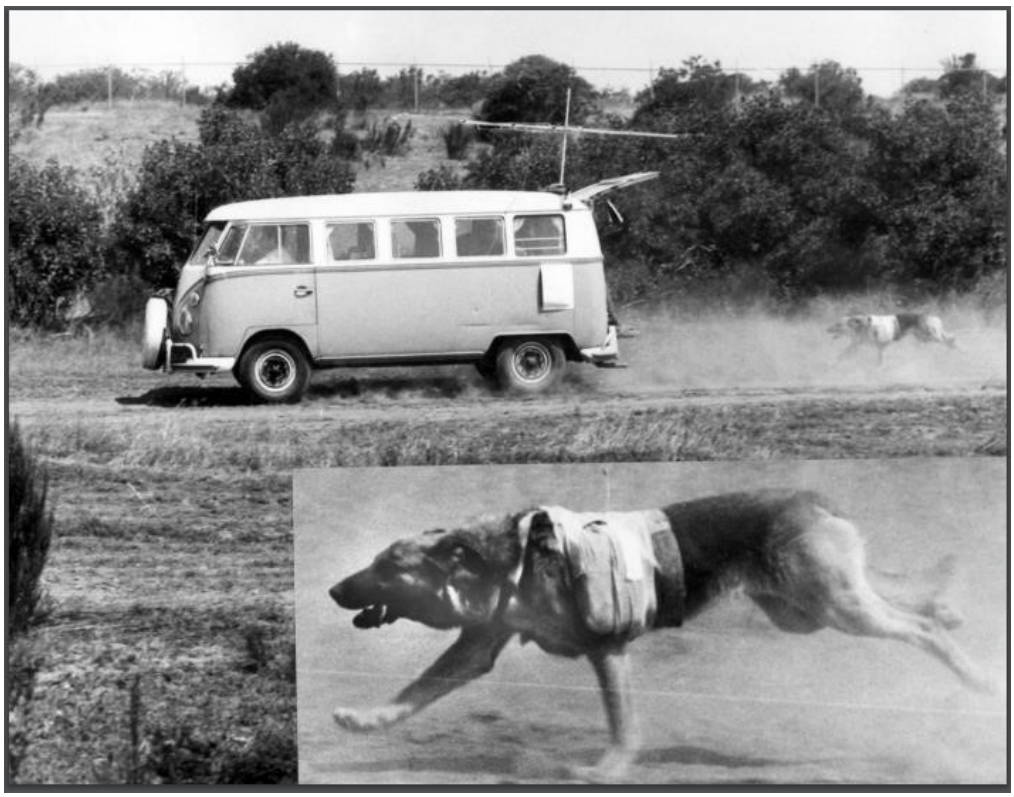
Direct pressure measurement has always been understood to be more accurate compared to the indirect non-invasive methods. The history of blood pressure measurement started with direct intra-arterial measurements by Revd Stephen Hales (Hales, 1733) and following nearly 300 years of development and refinement resulted in various telemetry systems where blood pressure can be measured continuously in groups of free-moving conscious animals (Knot and Lee, 2016).

Optical manometer originally developed by Otto Frank in 1903 (Geddes, 2002, 2013) and later work by Hamilton, Brewer and Brotman (Hamilton et al., 1934) enabled reliable recordings of the pulse contours, however this still lacked in accuracy and had other disadvantages for routine laboratory and clinical use such as rigid spatial constraints. Conversion of pressure signals to electrical energy was understood to help to overcome these limitations and thus resistance wires and strain gauge manometer were developed in the early 1940's (Lambert and Wood, 1947). The strain gauge pressure transmitter consisted of a balances Wheatstone bridge made of strain-

sensitive resistance wires mounted on a cantilever suspension. Pressure wave causes movement in the suspension that causes current to flow in the output circuit. The output current is directly proportional to the strain and thus the pressure that caused the strain. The recordings are made using galvanometer. The limitation of this system was low natural frequency compared to the optical manometer. Nevertheless, the technology paved the way to further progress in developing the devices that can sense pressure and volume changes (Grundfest et al., 1945; Lambert and Wood, 1947) that can be applied to direct and indirect blood pressure measurement.

A tethered system for rats was reported in 1956 (Still and Whitcomb, 1956) and later for mice (Van der Meer and Van Bekkum, 1959): a cannula is inserted into the carotid artery and exteriorised at the back to be connected to the condenser manometer (capacitive displacement meter).

The first physiological signal was telemetered from a conscious animal in 1963 when aortic blood flow was successfully transmitted from an exercising boxer dog at the San Diego Zoo hospital (Sarazan and Schweitz, 2009). It took nearly 30 years for the telemetry technology to become available for mice (Kramer et al., 2000; Mills et al., 2000).



Photograph 1.1. One of the earliest illustrations of telemetry equipment. *Classic exercise physiology studies conducted in San Diego, USA: the instrumented dog was encouraged to chase the vehicle containing the telemetry receiving and recording equipment by the person sitting in the rear hatch. Taken from 'The Story of The Development of Non-invasive Heart Care' as detailed in an American Physiological Society Press Release. http://www.the-aps.org/mm/hp/Audiences/PublicPress/Archive/09/38.html*

Table 1.3. Timeline of blood pressure measurement in animals *(focus on rodents)*

Year	Author/Inventor	Description and notes
1927	(Durant, 1927)	Blood pressure is measured in anaesthetised rats by cannulating abdominal artery under anaesthesia.
1933	Hamilton W.F., Brewer G. and Brotman I.	Pressure contours measured in intact small animals (mouse, rat, small birds) using optical manometer (Frank's capsule)
1934	Griffith J.Jr.	Indirect blood pressure measurement in anaesthetised rats by occluding blood flow in the foot and paw capillaries are observed as the cuff is released
1934	(Bonsmann, 1934)	Photocell apparatus (Visomat, Leipzig) is reported to be used in blood pressure recording in conscious white mice and rats.
1938	(Byrom and Wilson, 1938)	Plethysmographic method for measuring systolic blood pressure in anaesthetised rat
1939	Diaz J.T. and Sanford E. Levy	An indirect method for repeated determinations of blood pressure of anaesthetised rats by bleeding the tail.
1939	Williams J.R., Harrison T.R., Grollman A.	Tail-cuff plethysmography to measure blood pressure in conscious rat and mice
1941	(McMaster, 1941)	Estimation of blood pressure in the anaesthetised mouse by observing the disappearance and reappearance of circulation in the hind paw claw following occlusion of flow to that paw

Year	Author/Inventor	Description and notes
1945 - 1948	(Grundfest et al., 1945) (Lambert and Wood, 1947) Bierman H.R.	Wire resistance strain gauge is developed for intra-arterial blood pressure measurement and peripheral pulse recording in humans and animals
1949	Friedman M. and Freed S.C.	Microphonic manometer for indirect determination of blood pressure in conscious and anaesthetised rats
1949	Noble F.W.	Capacitance manometer
1949	Farrell G.L. and Anderson E.	Cuff method surgically placed around the abdominal aorta (and vena cava to avoid vessel injury during separation) of the rat
1954	Algire G.H.	Peripheral blood pressure in conscious restrained mice by surgically inserting a transparent chamber on dorsal skin.
1956	Still J.W and Whitcomb E.R.	Tethered system for direct blood pressure measurement in the conscious rat
1959	Van der Meer and Van Bekkum	Tethered system to measure blood pressure in conscious mouse
1963	Franklin and Van Citters	First telemetry signal transmitted
1963	Pressman and Newgard	Tonometry principle first described: Transducer for continuous external measurement or arterial BP
1988	Clement J.C. et al	Telemetry system to measure core temperature and activity in freely moving mice
1991	Brockway B.P. et al	Telemetry system for continuous chronic measurement and recording of blood pressure, heart rate and activity in the rat
1993	Kramer K. et al	Telemetry to record ECG and heart rate in freely moving mice
2000	Kramer et al	Telemetry for continuous chronic measurement and recording of blood pressure, heart rate and activity in the mouse
2016	Knot and Lee, 2016	Multi-channel telemetry system to allow group housing

1.4.8. Consideration of the 3Rs in measuring murine blood pressure

Progress in understanding how living things function rely on experiment using the living organisms. Some of the earliest advances in our understanding of human anatomy were made in the period when dissection of human corpses was allowed in Alexandria (Bay and Bay, 2010). The discovery of circulation, the development of the techniques to measure blood pressure, the understanding of the mechanisms of blood pressure regulation – all of these and most others – relied on the experiments with live animals. The Age of Reason in physiology is synonymous with experiment in living animals (Hales 1732, Hedrich 2004, Henry 1965) and the use of animals in the laboratory was established by 1860s (Rowan, 2007).

In-vivo science touches on a fundamental question of the relationship of humans and animals and poses a range of moral dilemmas. Therefore, the use of animals in scientific procedures has been matter of heated debate since the very beginning both from outside and within the scientific

community. The established routine use of animals in the laboratory in Great Britain in the middle of the 19th century brought about near immediate reaction from the public that resulted in the first in the world legislation that regulated the use of animals in scientific procedures (Hamilton, 2016). The Cruelty to Animals Act 1876, (also known as the Vivisection Act or Charter) came to replace the Act to Prevent the Cruel and Improper Treatment of Cattle ("Martin's Act") in the specific issue of using animals in scientific experiments. It became necessary to have a personal licence to carry out such experiments and a system of inspections of the facilities was also put in place in the UK. The Act regulated the use of vertebrate animals, putting some restrictions on how the animals could be used, and also made it obligatory to report the numbers and species used in the experiments. Sentience, attributed to the presence of complex nervous system that vertebrate animals all have in common, was the underlying principle for the protection given to these animals. Since then, the use of animals in the UK has been under public and government scrutiny.

The Cruelty to Animals Act 1876 was replaced by the Animals (Scientific Procedures) Act 1986 [A(SP)A] that (was further amended in 2013). More complex regulations and more vigorous checkpoints to justify the use of animals in scientific procedures were put in place. Higher protection status was granted to cats, dogs, horses and non-human primates as a consequence of the new legislation; octopus was added to the list in 1993 of the protected animals on the grounds of high level of sentience and cognitive capacity displayed by this species. The policy and concepts regarding the use of animals in research evolve continuously. The result of these developments was the 3Rs policy (Russell et al., 1959) that governs all the research that involves animals today. The 3Rs stand for Replacement, Reduction and Refinement:

Table 1.4. 3Rs principles: definitions

	Standard	Contemporary
Replacement	Methods which avoid or replace the use of animals	Accelerating the development and use of models and tools, based on the latest science and technologies, to address important scientific questions without the use of animals
Reduction	Methods which minimise the number of animals used per experiment	Appropriately designed and analysed animal experiments that are robust and reproducible, and truly add to the knowledge base
Refinement	Methods which minimise animal suffering and improve welfare	Advancing research into animal welfare by exploiting the latest *in vivo* technologies and by improving understanding of the impact of welfare on scientific outcomes

Adapted from https://www.nc3rs.org.uk/the-3rs

The number of animals used in scientific procedures rose steadily following the end of the Second World War, reaching its peak (over 5.5 million) in the 1970s, thereafter steadily declining until the development of gene editing tools in the vertebrates in the early 2000s. Genetically altered animals fuelled the increase in the procedures until 2013. More recently, the total number of procedures on animals declined in 2018 compared to both the previous year and 10 years before (Annual Statistics of Scientific Procedures on Living Animals Great Britain 2018, (Home Office).

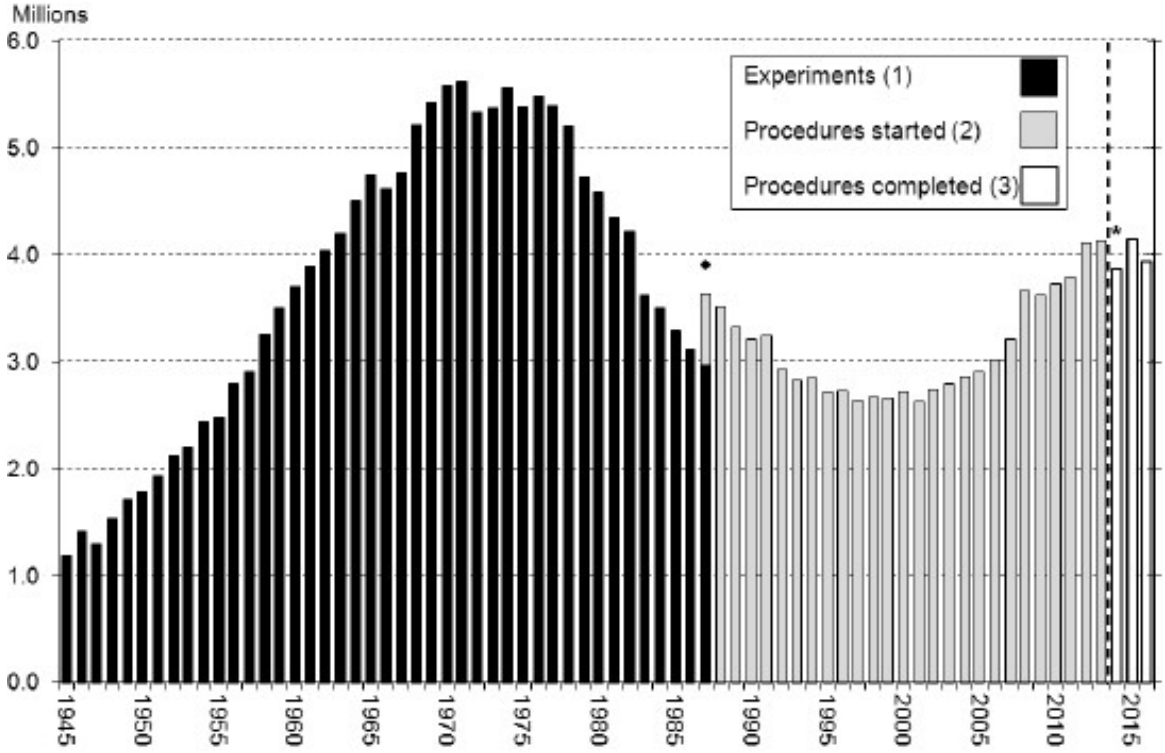


Figure 1.7. Total experiments / procedures on live animals in the years between 1945 and 2016. Source: the Annual Statistics of Scientific Procedures on Living Animals Great Britain 2016 (Home Office).

Chart notes:
(1) Experiments **started** under the Cruelty to Animals Act 1876.
(2) Scientific Procedures **started** under the Animals (Scientific Procedures) Act 1986.
(3) Following the transposition of European Directive 2010/63/EU into UK law, scientific procedures **completed** under the revised Animals (Scientific Procedures) Act 1986.
◆ The 1987 total includes experiments started under the 1876 Act as well as procedures started under the 1986 Act.
* The data collection methodology changed in 2014 which resulted in some under-reporting for that year (see section on changes to data collection from 2014 in the user guide).

A substantial portion of cardiovascular research is carried out in vitro using human and animal vascular cells. However, animal models are an essential tool in order to learn of whole-body integration of novel mechanisms involved in the onset and maintenance of hypertension. They have been developed based on the etiological factors which are responsible for human hypertension.

Angiotensin II (Ang II)-induced hypertension in the mouse is one of the most common hypertensive models used to date, to mimic the central role of Ang II in human hypertension. Medicines that either block the formation of Ang II, or antagonise its actions have a primary role in the treatment of hypertension, but despite this, the cardiovascular risk for patients remains high and the mechanisms involved in Ang II-induced hypertension are very complex. It is common for the disease to escalate and it is now realised that less well understood factors, such as vascular dysfunction, inflammation and oxidative stress play significant roles, that are poorly understood (Paulis and Unger 2011). The Ang II-induced murine hypertensive model is a valuable research technique. It is used to investigate/evaluate mechanisms as well as potential and newly modified therapies, often involving genetically-modified mice. For example, there are approximately 200 manuscripts published in the last 12 months that report the use of Ang II – induced model of hypertension in the mouse, i.e. over 1,500 mice were used in this protocol.

Cardiovascular biology research and the use of such models as the Ang II hypertensive model heavily relies on the accurate and reliable blood pressure measurement in conscious mice. The technologies for both invasive and non-invasive blood pressure monitoring have evolved since these were first described in the use in rats (Williams et al., 1939; Buñag, 1983; Kramer et al., 2000; Kurtz et al., 2005a), however some of the issues that were originally highlighted still remain. Implantable radio-telemetry (later referred to as telemetry) devices measure blood pressure directly from the aorta, while the currently used tail-cuff sensor technologies, such as Volume Pressure Recording (VPR) and Photo Plethysmography, rely on detection of either flow or pulse following the occlusion of the tail artery, thus measuring the blood pressure at a peripheral site. Both invasive and non-invasive techniques are widely used in the mouse. Whilst telemetry is a direct measurement of central blood pressure and is considered the gold standard technique (Kurtz et al., 2005a), it requires the mice to undergo major surgery for the implantation of the telemeter probes and subsequent recovery. The probe implantation requires permanent ligation of an artery, most commonly a left carotid, in mice, which is a significant perturbation to the circulation (Polycarpou et al., 2016) and the weight of the probe can compromise the animal's welfare (Einstein et al., 2004). Moreover, the telemetry equipment is expensive, and the animals are often singly housed (compromised welfare) or have a companion animal (increases the number of animals used). By comparison, the tail-cuff does not have these limitations and the use of the tail cuff technologies is widely considered to be acceptable and recommended for certain applications such as phenotypic screening and studies that involve large numbers of mice (Kurtz et al., 2005a; Feng et al., 2008). Despite being associated with restraint stress, some consider the tail cuff technique to be more acceptable for the 3Rs' term of refinement because it is performed without the need for anaesthesia or surgery.

Previous studies have compared tail-cuff sensor technologies with the invasive blood pressure monitoring in anaesthetised (Krege et al., 1995; Reddy et al., 2003) and conscious mice (Johns et al., 1996; Whitesall et al., 2004; Feng et al., 2008). Volume Pressure Recording (VPR) sensor technology for tail-cuff, presently one of the most commonly used, was validated by Feng et al (Feng et al., 2008). They found negligible difference between telemetry and VPR systems, however it was also noted that the agreement was not consistent across the whole range of blood pressure recordings. It was not made clear by them if the discordance between the two methods to measure blood pressure arose from the deficiencies in the tail-cuff sensor technology or the different physiology of the central and peripheral circulation.

1.5. Aims and Hypotheses

The hypothesis in this project is that we are able to improve the handling technique in a manner that will substantially reduce stress associated with the tail cuff technique.

Aims are as follows:

- Characterise the handling techniques that can potentially reduce stress response in the mouse.
- To develop a protocol to allow simultaneous measurement of mouse blood pressure by telemetry and tail cuff.
- To determine the impact of different types of handling as well as gender on tail cuff measurements as compared with simultaneous telemetry. This allows assessment of blood pressure and heart rate as markers of acute stress.
- To assess the effect of handling, restraint and warming on the cardiovascular parameters and core body temperature.
- To compare the readings of blood pressure obtained by the tail-cuff and telemetry in an Ang II-model of hypertension.

Chapter 2

Methods and Materials

2.1. Animals

In vivo experiments were performed according to the UK Home Office Animals Scientific Procedures Act 1986 and approved by King's College London Animal Care and Ethics Committee. ARRIVE guidelines were used for reporting the procedures (Kilkenny et al., 2010). Male and female C57Bl/6J mice used in these experiments had free access to food and water, were maintained in climatically-controlled environment at 22 ± 2°C humidity 50 ± 10% under filtered positive pressure ventilation on 12:12h dark : light cycle beginning at 07:00 GMT. All mice were singly housed following the implantation of the telemetry probes in standard sized Perspex cages with wood chip bedding, paper nesting material and cardboard enrichment tubes. All mice used in these experiments were bred in house. The age range of the mice at the start of the procedures was 12-15 weeks.

The following mice were used.

Chapter 3. Male CD1 (n=15) and C57Bl6/J male (different groups of a total of 22 mice) and C57Bl6/J female (n=7) mice were included in the studies to characterise the tail-cuff protocol and the handling studies that only used the tail-cuff technique. Male CD1 mice were bought from Charles Rivers (UK) and were allowed to acclimatise in the animal holding facility for one week. Age range of the CD1 mice used in these experiments was 12 -13 weeks. All C57Bl6/J mice used in these experiments were bred in house and the age range of the mice was 12-14 weeks at the start of the experiments. Additional six male C57Bl/6J mice were used in the further telemetry-based experiments. The age range of the mice used in these experiments at the start of the procedures was 15 - 16 weeks.

Chapters 4 and 5. Further stages of the project relied on telemetry-based experiments and only C57Bl/6J mice were used. A total of 8 male mice and 12 female mice were implanted with blood pressure telemetry (PA-C10, DSI) probes. Additional 4 female mice were implanted with temperature telemetry (TA10TA-F10, DSI) probes. Age range of mice used in these experiments ranged between 14-15 weeks at the start of the experiments. Typically 4 – 8 mice were implanted at a time for each individual experiment. In some cases the data from the several such experiments was combined to include the data from the 6 male C57BL/6Jtelemetry-impanted

mice used in the experiments described in chapter 3. The details of experimental design for each stage of the experiment will be further outlined in each chapter.

2.2. Surgical procedures

All procedures were conducted using aseptic techniques under isoflurane anaesthesia (Abbott Laboratories, UK); anaesthesia was typically induced using 4% isoflurane and maintained at 2% in 2l/min O_2. Buprenorphine was administered perioperatively (50µg/kg, i.m., Vetergesic, Sogeval UK ltd) for pain relief. All animals were supplied with soft diet following surgery.

2.2.1 Implantation of radio-telemetry probes to measure blood pressure

Blood pressure, heart rate and activity were measured using radiotelemetry [PA-C10; Data Science International (DSI), St. Paul, MN, USA]. The catheter of the blood pressure transducer was inserted in the left carotid artery and advanced towards the aortic arch. The catheter was secured using surgical braided silk (5.0, waxed, Pearsalls sutures, Pearsalls Ltd, UK) and the transmitter was placed s.c. in the right flank. The transmitter pocket was irrigated with sterile saline (0.9% saline; sodium chloride, pyrogen free) and the outer wound closed with absorbable sutures (5.0, Ethicon, Johnson and Johnson) in a discontinuous pattern (Marshall et al., 2013). All animals were singly housed and allowed to recover for 7-10 days before baseline recording of blood pressure or temperature.

2.2.2. Implantation of radio-telemetry probes to measure core body temperature

The abdomen was shaved and wiped with surgical iodine. A small ventral midline abdominal incision (<1 cm) was made, and the abdominal muscle wall was exposed. A ventral incision was made on the abdominal wall and was irrigated with sterile saline (0.9% saline; sodium chloride, pyrogen free) to facilitate the insertion of the radiotelemetry transmitter (TA10TA-F10; DSI, St. Paul, MN, USA]. Following implantation, the abdominal wall and the skin incision were sutured separately using absorbable sutures (Vicryl 4.0; Ethicon, Johnson & Johnson, New Brunswick, NJ, USA) (Alawi et al., 2015). All animals were singly housed and allowed to recover for 7-10 days before baseline recording of blood pressure or temperature. Following the recovery period the core body temperature and activity were recorded either continuously or at 1-10 min schedule intervals depending on the type of experiment.

2.2.3. Implantation of Angiotensin II (Ang-II) osmotic mini-pump

Osmotic mini pumps (1002; Alzet) were surgically implanted containing Ang-II (Sigma, UK) at a dose of 1.1mg/kg/day or saline (control) for 14 days, as previously described (Smillie et al., 2014).

To implant the mini-pumps, mice were anaesthetised using isoflurane. Dorsal skin around the scapular region was shaved and cleaned using isopropyl alcohol and surgical scrub. Small incision in the skin was made and further blunt dissected to widen the incision (up to 5 mm wide) site and subcutaneously towards the caudal region. The subcutaneous pocket was flushed with warmed sterile saline and the filled mini-pump was inserted into the subcutaneous pocket towards the tail. The incision site was closed using absorbable sutures (Vicryl 4.0; Ethicon, Johnson & Johnson, New Brunswick, NJ, USA) in a discontinuous pattern.

2.3. Angiotensin-II (Ang-II) murine hypertension model

Only mice implanted with blood pressure telemetry transmitters (as described in section 2.2.1) were made hypertensive using Ang-II in the studies included in this project. These mice were also subjected to the tail-cuff technique (as described in section 2.5.1) and had their blood pressure measured by telemetry and the tail-cuff, including simultaneous telemetry and the tail-cuff recording (as described in section 2.5.3). Prior to being implanted with telemetry devices, the mice were exposed to the tail-cuff protocol as described in section 2.5.1 in order to habituate them to the technique. Telemetry transmitters were implanted as described in section 2.2.1. Following at least 7 days recovery, baseline (normotensive) blood pressure recordings were obtained over at least 48 hours using telemetry. Baseline (normotensive) measurements by the tail-cuff were made on at least two occasions after the baseline measurements by telemetry were completed. Thereafter the animals were surgically implanted with osmotic mini pumps (1002; Alzet) containing Ang-II (Sigma, UK) at a dose of 1.1mg/kg/day or saline (control) for 14 days, as described in section 2.2.3. Blood pressure monitoring by telemetry was resumed. Tail-cuff plethysmography was additionally carried out on days 3, 5, 9 and 11 following mini-pump implantation and compared to telemetry recordings acquired at the same time by and to those obtained by telemetry before the animals were disturbed for the tail-cuff technique.

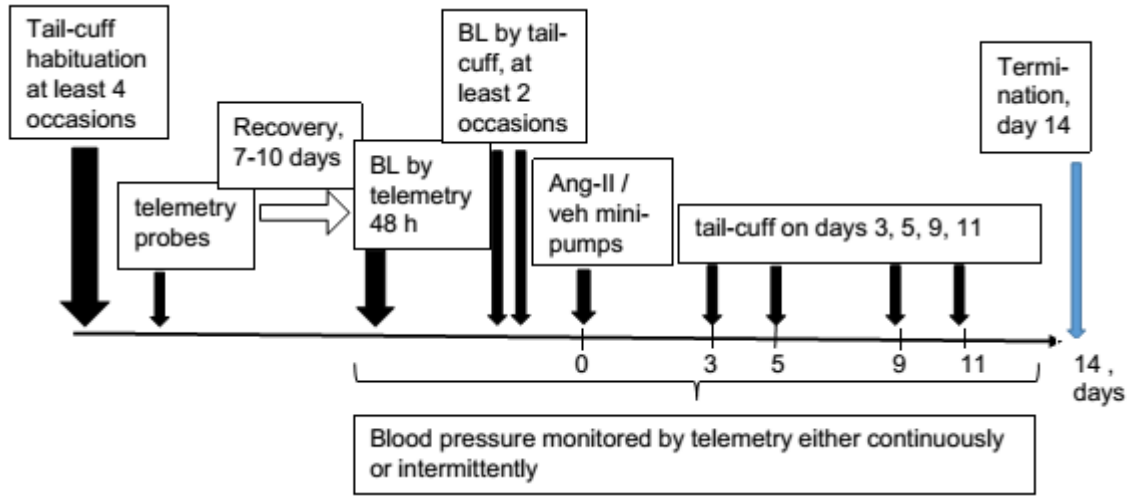


Figure 2.1. The experimental procedures and their timings used in the hypertension model. BL=baseline - refers to the measurements made by telemetry in undisturbed mice, and also to the measurements by the tail-cuff before the implantation of Ang-II or vehicle mini-pumps. Simultaneous blood pressure recordings by telemetry and the tail-cuff were carried out on at least two occasions before the mice were implanted with the Ang-II or vehicle mini-pumps and thereafter on days 9 and 11 for study number 4, and on days 3, 5, 9 and 11 for the study number 5.

Baseline is a widely used term in physiology research and refers to the measurement made under resting or minimal activity conditions against which a change in the measured parameter can be evaluated (Jennings et al, 1992, Fishel et al, 2007). In the context of the experiments where measurements were made by telemetry alone, the term baseline is used to denote physiological measurements made in undisturbed mice. In the context of the experiments when only the tail-cuff system was used to measure the cardiovascular parameters, the term baseline was used to refer to the measurements made in mice once they are considered habituated to the tail-cuff technique as a reference against any change that may result from Ang-II infusion or other interventions.

2.4. Measuring blood pressure

2.4.1. Tail-cuff plethysmography

CODA 8 non-invasive blood pressure acquisition system for mice (Kent Scientific, Torrington, CT, USA) was used for all non-invasive blood pressure measurements. This system uses Volume Pressure Recording (VPR) to detect blood pressure based on volume changes in the tail.

Figure 2.2. The CODA Non-Invasive Blood Pressure System*. A) CODA High Throughput system for 8 animals: 1 – the CODA controller with 8 occlusion cuff and 8 VPR port connectors as indicated on the diagram , 2 – computer connected to the CODA controller via a USB cable, 3) two heating pads with 8 animal holders. B) Photo showing rear part of the mouse placed into the mouse holder and the illustration of the arrangement of the occlusion cuff (O-cuff) and the VPR cuff on the mouse's tail. (Image taken from https://www.kentscientific.com/Customer-Content/www/products/Files/CODA_HTManual.pdf)*

Although the detailed information on the method the system uses for pressure measurement is not available as it is proprietary information, the overall process can be described as follows. The VPR cuff inflates first, pushing blood out of the tail. Next, the occlusion cuff inflates to approximately 250 mmHg to prevent flow of blood to the tail. The VPR cuff releases, and then re-inflates partially so that it fits closely to the tail. The tail is thought not to contain any significant amount of blood at this point. The occlusion cuff begins to deflate and blood flow returns to the tail. The tail volume is increasing as a consequence and pushes against the VPR cuff bladder. This tail swelling can be observed as the positive slope in the blue line (see figure 2.3). It is over this time the systolic and diastolic blood pressure are measured by the tail-cuff system. When the pressure in the occlusion cuff falls low enough not to obstruct the blood flow into the tail, the tail volume increases and VPR cuff is fitted fairly tightly on the tail and is able to detect the animal's pulse. The tail pulsations are displayed on the graph as vertical markers at the end of the measurement cycle. Figure 2.3 below shows the software output for one measurement cycles.

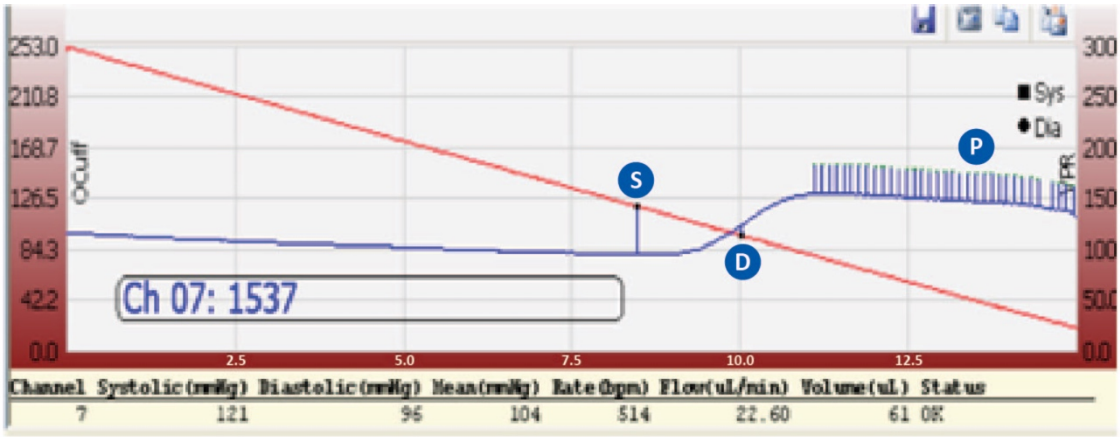


Figure 2.3 Example of CODA software output (further annotated) for one measurement cycle*. Left Y-axis represents the pressure in the occlusion cuff, units: mmHg. Right Y-axis represents the pressure in VPR cuff, units: mmHg. Red trace represents the pressure change in the occlusion cuff that was set to deflate over 20 seconds. Blue line represents pressure change in the VPR cuff. The X-axis shows the time, in seconds. The bottom panel shows the measurements generated during the measurement cycle. Point* S *is where the pressure in the VPR cuff begins to rise and is linked to SBP. Point* D *is where the pressure change in the VPR cuff has reached its maximum value and is linked to DBP. Vertical lines* P *on the VPR pressure trace represent pulse.*

The measurements made by the tail-cuff require sufficient blood flow in the tail that will allow to achieve adequate volume increase in the tail and thus supposedly enable more reliable measurement of the systolic and diastolic blood pressure based on the volume changes in the tail as detected by the VPR cuff. This adequate blood flow to the tail is typically achieved by warming the mouse to at least 32°C during the sessions. Great care must be taken not to overheat the mouse. The recommended tail temperature range is between 32 and 35°C. It is recommended that the ambient temperature in the room where the measurements take place should be at least 26°C. The heating platform is designed to achieve the required warming of the mouse during the sessions. It has heating settings "1 to 6" to help achieve the optimal tail temperature. Setting "3" is recommended as standard for ambient temperatures at around 25°C, while setting "4" and above may be necessary for lower temperature. Ambient temperature of 22°C or below is not recommended for the procedure. Warming "blanket" is also useful to fine-tune the temperature. Monitoring the surface temperature of the tail and the mouse holding tube using a hand-held thermometer is also highly recommended.

It is manufacturer's recommendation that the minimum volume change should be 15µl (Daugherty et al., 2009). Low volume increase in the tail typically result in unreliable pressure measurements, which tend to be below of the expected for a normal mouse. Figure 2.4 illustrates some examples when low blood flow to the tail was achieved and no minimum acceptable volume change was set for the sessions.

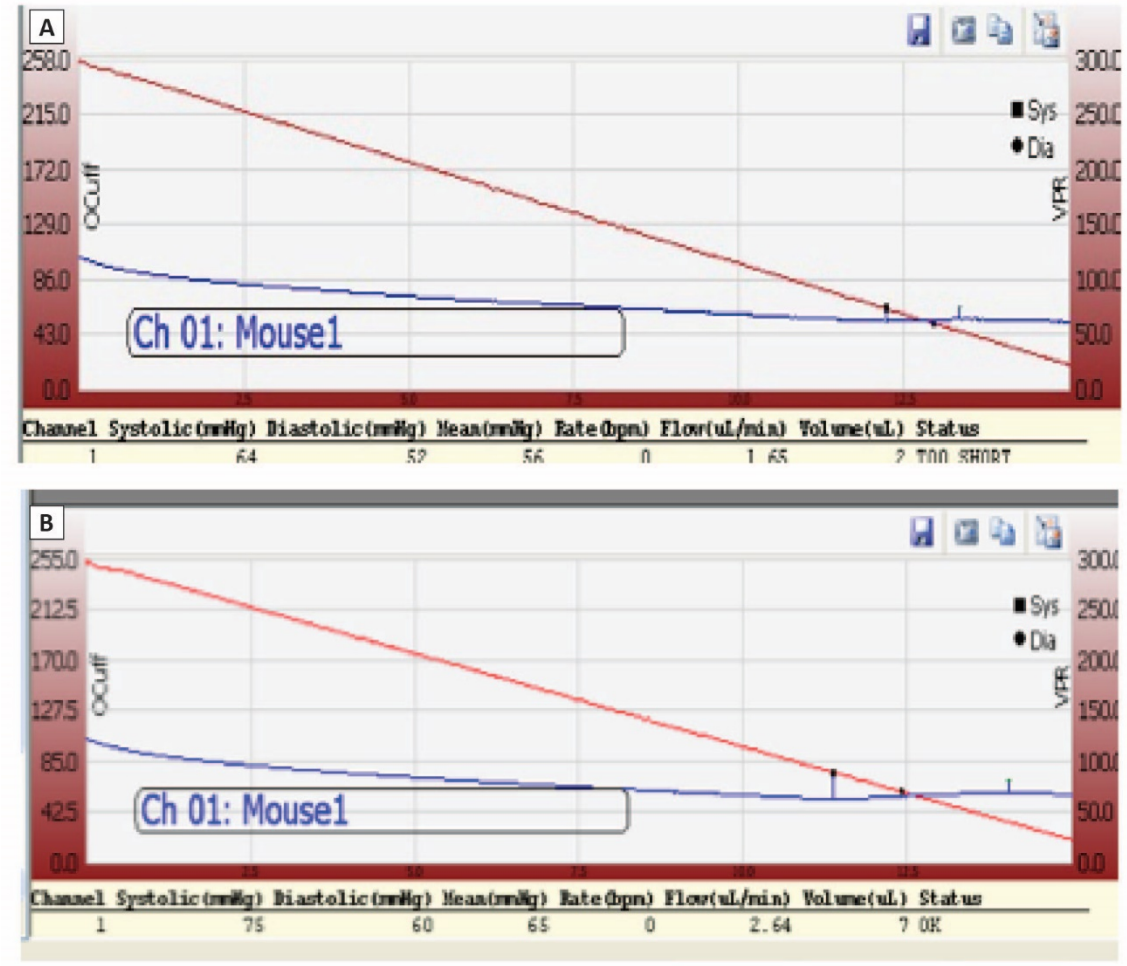


Figure 2.4. Tail-cuff VPR system output during the sessions when the measured tail volume fell below 15 µl. No minimum tail volume to accept the readings was set for these sessions. A) Example of a trace when the software has rejected based on insufficient pulse pressure; B) Example of a trace when the software has accepted the cycle, however the pressure readings are fairly low.

Any mouse movement during the measurement period that also affect the VPR cuff may create artefacts that may or may not be identified by the system. Figure 2.5 below illustrates the cycles most likely affected by movement.

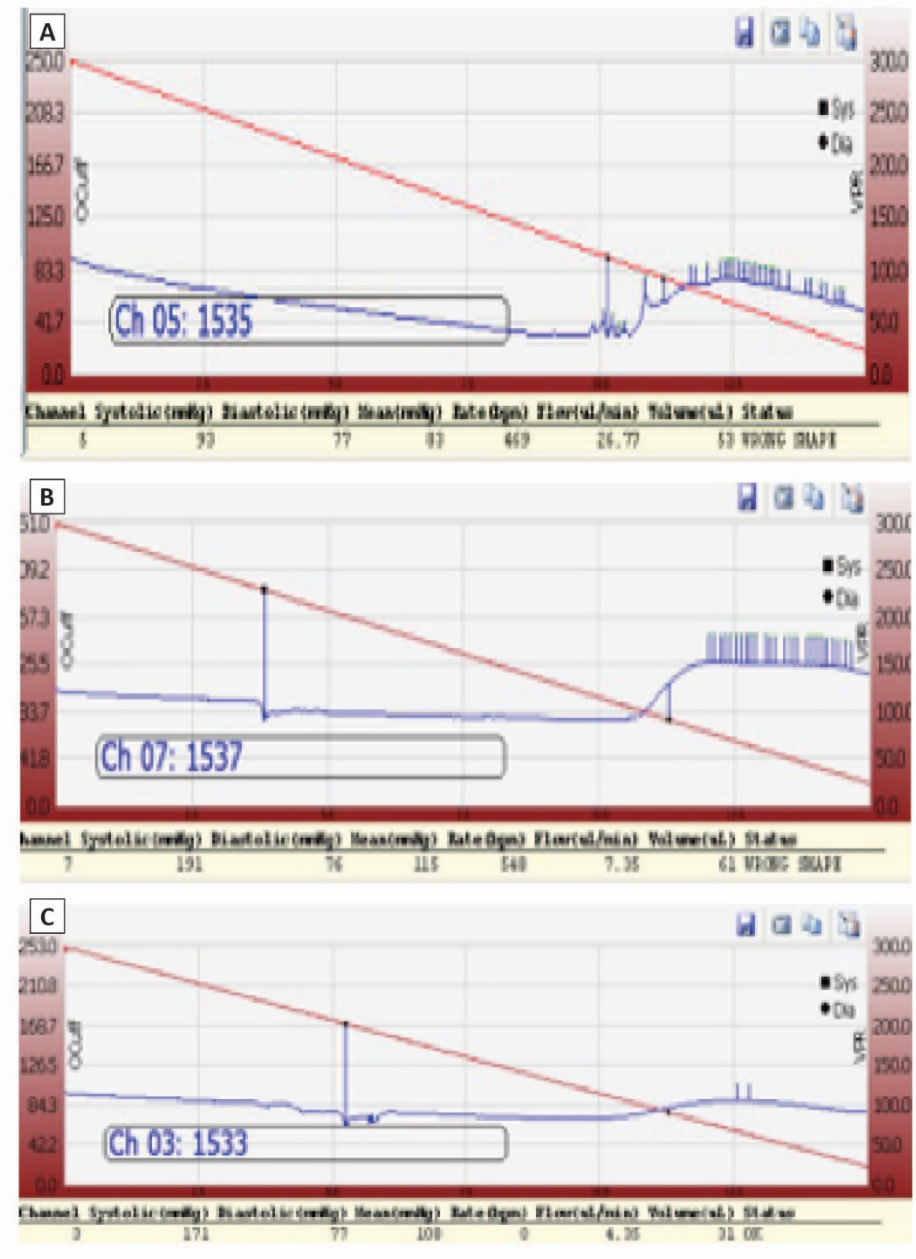


Figure 2.5. Examples of CODA software outputs for the measurement cycles most likely affected by mouse movement. A) Cycle rejected by the software; B) cycle rejected by the software. Most likely mouse movement was responsible for the perturbation in the VPR trace (arrow) and the consequent high measured systolic blood pressure; C) similar perturbation most likely occurred as in trace (B), however the cycle was accepted by the software.

The software has an algorithm to reject the artefactual readings based on a range of parameters. The software makes an assessment based on the shape of the VPR trace. Furthermore, if the measured pulse pressure (the difference between the systolic and diastolic pressures) is less than 14 mmHg, these readings are typically rejected.

The following settings and experimental conditions were used. The blood pressure measurement experiments were conducted in a designated area for behaviour experiments or otherwise quiet room where the test animas were normally held. Typical temperature range of these rooms was 22-25°C. I typically used setting "4" or "3" on the warming platform, as well as the warming blanket to achieve the required temperature in the mouse's tail. If the experiments were conducted not in the animal holding room, i.e. the mice were transferred to the procedure room, the mice were left to acclimatise in the procedure room for at least 30 minutes before the tail-cuff protocol was initiated.

The CODA controller was switched on at least 30 minutes before use. The occlusion and VPR cuff patency was tested before each experimental session and the cuff bladders for any cuffs that failed were replaced. Heating pads supplied as part of the CODA 8 system were preheated to 33-35°C (setting "3" on the heating platform was typically used).

The mice were placed into the holding tubes and the space was adjusted to minimise movement of the mouse whilst in the tube, at the same time taking care that the restraint does not cause pain or restricts mouse's breathing. The occlusion cuff was placed at the base of the tail and the VPR sensor cuff was placed adjacent to the occlusion cuff (as shown in figure 2.2). The mice were typically covered with the warming blanket that was supplied as part of the kit to help retain the heat from the platform. The mice were thus warmed up and acclimatised to the restraint for 5 minutes before the recording session was started. Surface temperature of the mouse and the surrounding surfaces was monitored during the recording sessions using TW2 infra-red thermometer (Thermoworks, Utah, USA). If the measured temperature exceeded 36°C, the warming blanket was removed and the heat setting on the platform was reduced if necessary. If the temperature was below 32.5°C, the warming platform setting was increased. Setting "4" was the maximum setting used and could be achieved by holding down the heat setting button for several seconds.

To measure blood pressure, the occlusion cuff was inflated to 250 mmHg and deflated over 20 seconds. The VPR sensor cuff detected the changes in the tail volume during the occlusion cuff deflations and the minimum volume change was set as 15µl. The session consisted of 20-25 cycles, of which the first 5 cycles were acclimation cycles. The measurements obtained during the acclimatisation cycles were discarded and the measurements obtained during the subsequent cycles were used to calculate the average value. The results of the measurement cycles that were rejected by the software were automatically excluded. I further reviewed the traces for the measurement cycles to exclude other readings when a movement artefact was suspected (similar to example shown in figure 2.5C).

At the initial stage of characterising the tail-cuff protocol mice were trained for at least 14 consecutive days prior to baseline blood pressure measurements following the previously established protocol (Smillie et al., 2014). This was reduced to between 3-5 training sessions on consecutive days.

The results of the measurement cycles that were rejected by the software were excluded from further analysis. The traces for software-accepted measurements cycles were further reviewed and the traces such as those shown in figure 2.4C were further excluded. This additional step was targeted at the apparent movement artefacts that may be missed by the software algorithm.

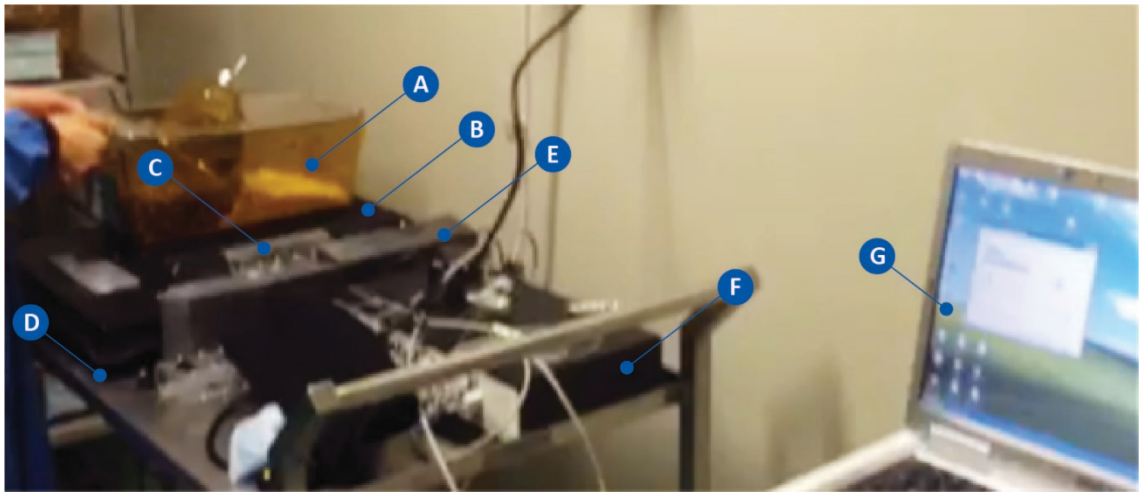
2.4.2. Radio-telemetry

PA-C10 (DSI) probes used in this study have high frequency solid-state pressure catheters that use non-compressible fluid to transmit the pressure signal from the lumen of the artery (the aortic arch) to the sensor within the transmitter body. The pressure wave in the artery is sampled at 1000Hz frequency. Ponemah v.5.10 software captured the pressure wave and analysed this data to show systolic, diastolic, pulse pressure, mean arterial pressure and heart rate.

Following the recovery period, blood pressure, heart rate and activity were recorded either continuously or at 1-10 min intervals depending on the type of the experiment. Baseline recordings over at least 36-hour period were obtained for all animals.

2.4.3. Simultaneous telemetry and tail-cuff blood pressure recording

Telemetry blood pressure was acquired at the same time as tail-cuff recordings in the same animal by placing the telemetry receiver pad adjacent to the tail-cuff device. The arrangement of the equipment can be seen in the photo 2.1.



Photograph 2.1. Equipment arrangement for simultaneous telemetry and tail-cuff recordings. *Annotations from left to right: A) Mouse home cage, B) Heating platform, C) Tail-cuff mouse holder, D) Telemetry receiver – concealed underneath the heating platform - not seen on the photo, location indicated by the arrow, E) Lead blocks arranged between the telemetry receiver pad and the tail CODA controller, F) CODA controller, G) Computer connected to the CODA controller. Telemetry data was collected onto another computer, which is not shown in this photo.*

Computer clocks on the telemetry and tail-cuff systems were synchronised so that 2 or 10 -second segments of telemetry recordings were acquired throughout the duration of the recording by tail-cuff. To compare the readings obtained by the two systems, average reading obtained using tail-cuff for each session were calculated. They were compared to the average value calculated for data collected by telemetry during the same period for the same animal. To establish the correlation between the two techniques, individual readings obtained by the two systems were aligned and compared (approximate temporal resolution 2-5 seconds).

2.4.4. Non-simultaneous telemetry and tail-cuff blood pressure recordings

To compare blood pressure readings obtained by telemetry when the mice were neither restrained nor otherwise stressed to those obtained by tail-cuff, we used blood pressure recordings obtained on the same day for each mouse before handling. Typically, a 0.5–1-hour period was chosen when the mouse was minimally active 0.5-2 hours before the mouse was handled for the tail-cuff recordings. The average for systolic and diastolic pressure readings obtained by telemetry over this period was calculated and compared to the calculated average to the systolic and diastolic pressure readings obtained by the tail-cuff on the same day.

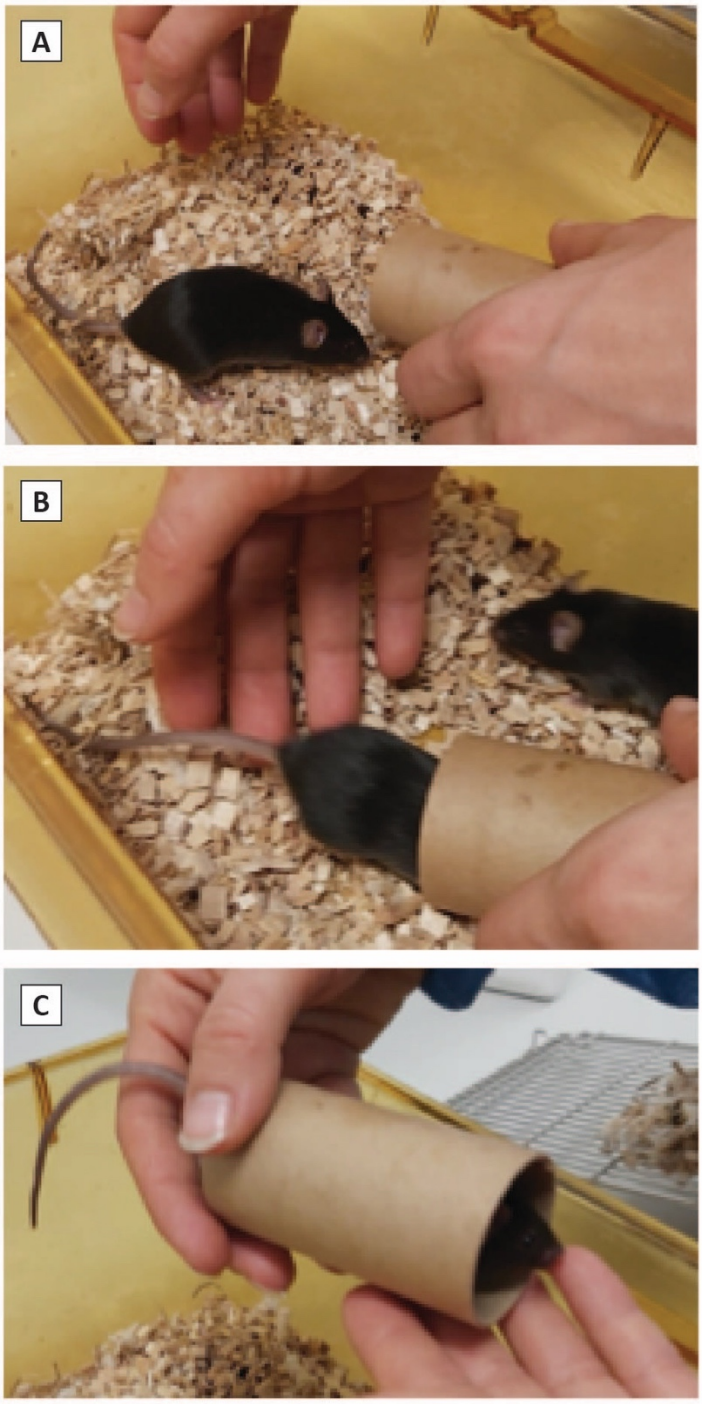
2.5. Measuring core body temperature using telemetry

Radiotelemetry transmitters (TA10TA-F10; DSI, St. Paul, MN, USA] were used for measuring core body temperature and activity. Ponemah v.5.10 software captured and analysed the signal from the temperature sensor and activity counter. Following the 10-day recovery period, core body

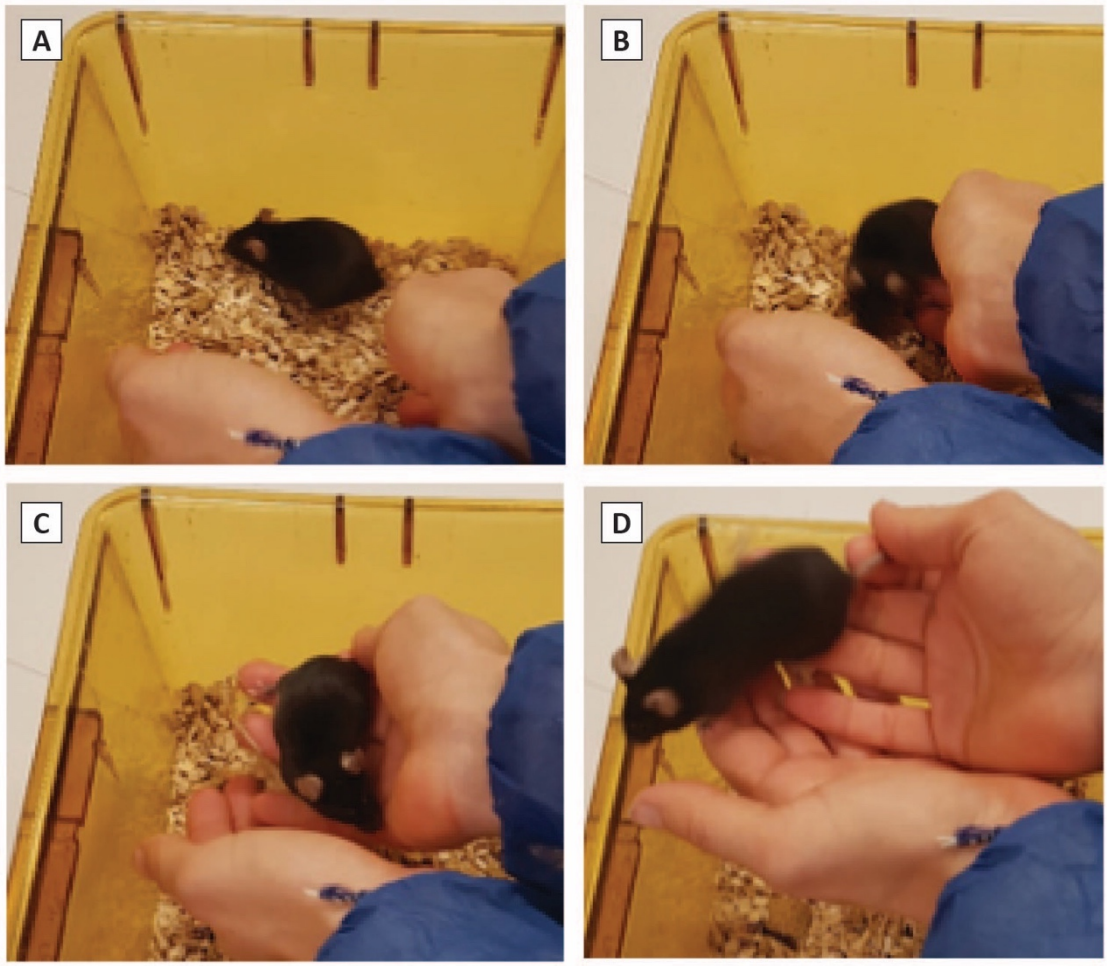
temperature was measured over approximately 36 hours using 10 seconds recording scheduled every 10 minutes (schedule recording). Continuous recording was used to record during the experimental procedures, in which cases the software was set to produce the output as average values over 10-second, or in some cases 1-minute, intervals.

2.6. The effect of different handling techniques on acute blood pressure during the tail-cuff protocol

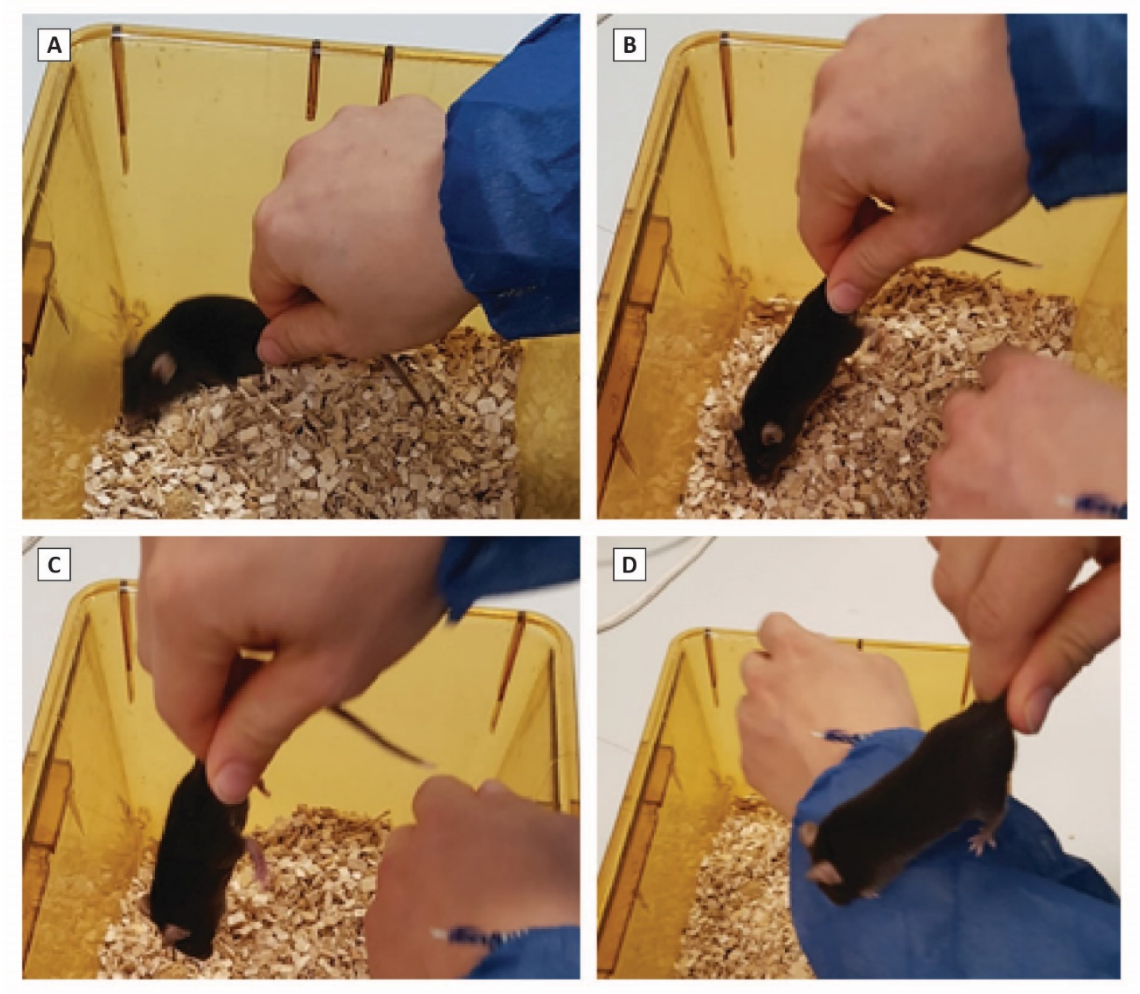
The following three common mouse handling techniques were defined and tested based on a previous publication (Hurst and West, 2010): tube, tail and tail cup. Although "cupping" technique was originally described, I found it less consistent to perform, as also confirmed in later studies by Gouivea and Hurst (Gouveia and Hurst, 2017). The "cupping" technique was replaced by the "tail-cup" as described below.



Photograph 2.2. Tube Handling. *The snapshots of the handling sequence are shown top to bottom A- mouse is approached with the handling tube and encouraged to walk into the tube, often by using the other hand to guide the mouse in, B- the mouse is walking into the tube, C- the mouse is lifted in the tube and the tube may be supported by both hands.*



Photograph 2.3. Tail-cup Handling. *The snapshots of the handling sequence are shown left to right, top to bottom A- mouse is approached and immobilised by the base of the tail, typically using the thumb and index finger, B- the mouse is encouraged to step into the handler's hand (typically the same hand that was used to immobilise the mouse), C- the mouse is lifted in the palm of the hand while still holding on to the mouse's tail, D- mouse is in the palm of the hand, allowed certain degree of movement.*



Photograph 2.4. Tail Handling. *The snapshots of the handling sequence are shown left to right, top to bottom A- mouse is approached and immobilised by the base of the tail, typically using the thumb and index finger, B- the mouse is lifted up by the base of the tail, C- the mouse is carried by the base of the tail, D- mouse is placed on the back of the other hand, while the mouse's movement is restricted.*

For the 'tube' technique, the environmental enrichment tube (normally present in their cage) was used. The mice were lifted from the home cage in these tubes and transferred into the tail-cuff restraint tube, with minimal handling. For the 'tail' technique, the mice were picked up by the base of the tail, then placed on the back of the hand and moved to the platform to before being transferred to the tail-cuff restraint tube. For the 'tail-cup technique, the animals were immobilised by the base of the tail and lifted up in the palm of the hand to the platform to be placed in the restraint tube.

Each mouse (total n=6) underwent each handling technique during the experiment in a semi-random manner. This was achieved by randomising each mouse to a handling technique on the first test week so that the 3 handling techniques were tested in the same period, then again in the second week (ensuring the technique was different to the one used in the first week). In the

third week the remaining handling technique for each mouse was used. Each test period was for 5 days, with a rest period (6 days) in between (figure 3.2 in chapter 3).

To study the acute effect of each handling technique on blood pressure and heart rate within the tail-cuff protocol, the tail-cuff protocol was arbitrarily subdivided into the following steps: "baseline" (the period before the animals were disturbed), handling (typically 10-30sec), placing the animal in the restraint tube (30-60sec), acclimatisation in the restraint tube (typically 5 min), tail-cuff recording cycles. Telemetry was used to monitor blood pressure and heart rate during the experiment.

2.7. The effect of handler's gender on the mouse blood pressure and heart rate during the tail-cuff protocol

Tail-cuff and telemetry simultaneous recordings were carried out as described above. Each researcher handled 3 mice by their preferred technique (techniques used were recorded). Male and female researchers alternately carried out the recordings on consecutive days.

Time periods for picking up the mouse, placing in the tube, restraint in the tube, the start and finish of the tail-cuff recording session were recorded and average values for BP and HR were calculated for each animal.

2.8. The effect of heating and handling interventions involved in the tail-cuff technique on blood pressure and core body temperature

The following factors and steps are associated with obtaining tail-cuff measurements: presence of the researcher in the room, moving the mouse in the cage close to the equipment, placing the mouse in the restraint tube, heating and finally measuring blood pressure by the tail-cuff. Telemetry was used to dissect the effect of these interventions on the mouse central blood pressure, heart rate and core temperature.

The following experimental procedures were performed typically on separate days repeated on at least 2 occasions for each animal. The researcher would enter and remain in the room for approximately 1-5min period. The cage is moved close to the platform, without handling, or removal of mouse from the cage, where it stayed for 15 min. To test the effect of handling, the animals were picked up from the home cage and held in the hand for approximately 30 seconds and then returned to the home cage for further recording over 15 minutes. For the effect of restraint without heating, the mice were removed from the home cage and placed in the restraint

tube without pre-heating the tube or the underlying platform. To test the effect of restraint and heating, the mice were placed into the restraint tube and the platform pre-heated to 33-35°C. The animals remained in the restraint with or without pre-heating for 15 min. In both cases the restraint period lasted 15 minutes. The final step involved the tail-cuff procedure. For this the mice were removed from the home cage, restrained on the pre-heated platform as above and subjected to recording by the tail-cuff after 5 min of acclimatisation on the platform. Each recording session consisted of 15 cycles of inflation and deflation of the cuff over the animal's tail. The telemetry data (temperature or cardiovascular) was collected continuously at 10 sec segments and presented as 1-minute averages following the time point of initial capture.

2.9. Statistical analysis

The following criteria and conventions have been used as the basis for the statistical analysis. Accepted error, α, was set at 5% be default and the study power was accepted as 80%. Sample size estimation were performed based on the following formula:

$$n = \frac{(\mathrm{Z}\alpha + \mathrm{Z}1 - \beta)^2\,\sigma^2}{(\mu 2 - \ \mu 1)^2}$$

Where Zα = 1.96 and is a constant set by convention for the accepted error of 5% for 2-tailed test; Z1-β = 0.84 is a constant set by convention for the accepted 80% power of the study; σ=standard deviation and μ2 - μ1 is the difference between the means.

Data in the manuscript is typically expressed as mean ± SEM or mean ± SD (as specified). GraphPad Prism v5.0 software was used to draw all graphs. Statistical analysis was also performed using GraphPad Prism v.5.0 unless specified otherwise; IBM SPSS Statistics 22 software was used for more complicated analysis; *p<0.05* was considered to represent a statistical significance. Statistical analysis was as described for each figure and typically included RM-ANOVA as most of the experiments used repeated measures in the same mouse. If the same parameter was measured in the same mouse on several days, only the average value was used for further analysis. Bland-Altman analysis was used to investigate the agreement between telemetry and the tail-cuff, however the difference of the measurements obtained by the two techniques was compared to the measurements obtained by the "gold-standard" technique, unlike the average as reported by others (Feng et al., 2008).

Pearson correlation was used to assess the correlation between the two measuring techniques and regression analysis was used to support the correlation analysis in terms of what proportion

of data can be explained by the fitted linear model. Correlation was considered strong for Pearson r> 0.7 (or – 0.7), medium for Pearson r between 0.7 and 0.5 (-0.7 and -0.5), weak for Pearson r< 0.5 and 0.3 (-0.5 and -0.3) and negligible at less than 0.3 (-0.3) (Mukaka, 2012).

Chapter 3.

The Effect of Handling and Other Interventions Associated with the Tail-Cuff Protocol on the Mouse

3.1. Introduction

Measurement of blood pressure in conscious animals is most desirable because of the significant effect anaesthetic agents have on the cardiovascular system. However, the stress response is the main confounding factor for blood pressure measurements in conscious laboratory animals. The mere presence of humans and most laboratory procedures have been shown to induce stress in mice and affect the cardiovascular parameters (Johnson et al., 1992; Gross and Luft, 2003; Balcombe et al., 2004; Batchu et al., 2015).

The major limitation of the tail-cuff technique is that it requires handling and restraint, which are known to be stressful to mice and have direct and immediate effect on the measured haemodynamic parameters (Gross and Luft, 2003; Balcombe et al., 2004; Batchu et al., 2015). This undermines the reliability of the results obtained with this technique and raises a lot of controversy with regards to the studies that use the tail-cuff.

Handling, as part of laboratory procedures on animals, appears to have come to the attention of the researchers about thirty years ago. It was shown that rats experience fever following handling that can be reduced by prostaglandin inhibition. Similar results were shown to occur in mice (Cabanac and Briese, 1992) and the phenomenon was described as “emotional” fever. Mice appeared to habituate to the handling and colonic temperature measurements evidenced by lower maximum core temperature reached on each subsequent test day. This fits in well with the recommendation that animals should be habituated for the tail-cuff protocol, however the increase in the colonic temperature remained appreciable following 17 repeated exposures (Cabanac and Briese, 1992).

More recently, Hurst and West (Hurst and West, 2010) showed that mice handled using a tunnel or by hand-cupping were less anxious than the mice handled by the tail over time. Following nine handling sessions, the mice handled by the tunnel and cupping were more willing to interact with the handler (measured before and after the handling session), defecated and urinated less during the handling session, displayed lower anxiety scores in the elevated plus maze test and, importantly, displayed less aversion to brief restraint than their tail-handled counterparts.

Although restraint cannot be eliminated from the tail cuff protocol, the handling element promises a potential avenue to understand how the tail-cuff technique could be improved in the light of the series of experiments conducted by Hurst and colleagues (Hurst and West, 2010; Gouveia and Hurst, 2013). Considering the propensity of stress and anxiety to increase blood pressure (Ulrich-Lai and Herman, 2009), we speculate that the reduction in the biomarkers of anxiety seen with non-aversive handling, i.e. using the handling tunnel or the tube, will also translate into lower blood pressure and heart rate than the one seen when the animals are handled by the tail. The handling by tube, tail and cup-handling described in this study are similar to those described by Hurst and West (2010), while the tail-cup technique is based on my personal experience. I shall also investigate if male handlers, as compared to female handlers, induce larger increases in blood pressure and heart rate in mice.

The study to characterise the effect of the handling techniques on blood pressure in the context of the tail-cuff protocol can be subdivided in two phases: tail-cuff only and telemetry based. The tail-cuff only study examined if the handling techniques described by Hurst and West (2010) (tube, tail and cupping) and the "tail-cup" technique I and some of my colleagues routinely used in the laboratory differ in their impact on blood pressure and otherwise reflect the quality of the recordings by the tail-cuff technique. This was also the phase when I learnt the tail-cuff technique and I made some other observations that will be reported as well.

It can be easily appreciated that the tail-cuff technique itself has impact on blood pressure and heart rate, as shown previously (Gross and Luft, 2003). Moreover, the tail-cuff technique does not allow measurement of blood pressure during handling and is only possible while the animal is restrained in the holding tube and warmed up to a temperature of approximately 33 – 35°C. Telemetry, on the contrary, offers the advantage of continuous monitoring at high temporal resolution of the cardiovascular parameters in mice. Having telemetry equipment available in our laboratory, it seemed imperative to use it to characterise the effect of handling on the cardiovascular system. Whilst handling is an important part of the tail-cuff protocol, there are other steps involved that are already known to be stressful to the mouse, such as restraint, or are potentially stressful, such as placing of the mouse into the tube and the cuff inflations. I hypothesised that mice respond to handling with increases in blood pressure and heart rate, and that better handling can lower this increase during the handling and during the later stages of the protocol. Telemetry is used to investigate what effect handling and other interventions associated with the tail-cuff protocol have on blood pressure and heart rate of the mouse during the time of the interventions and recovery period. This data can be further used to compare the two blood pressure measurement techniques and will be discussed in the subsequent chapters.

The limitations associated with using telemetry restrict the number of animals and the duration of an experiment, which will be the important considerations in the design of the experiments.

The goal of this study is to investigate the acute effect of the defined handling techniques on blood pressure and heart rate during the tail-cuff protocol. Aims were:

- To characterise the tail-cuff technique in terms of optimal settings, the effect of operator experience, mouse strain and gender.
- To characterise the influence of different handling techniques (using a tube, by tail-cup, by cupping or by picking up by the tail result on blood pressure measurements by the tail-cuff system.
- To further investigate the effect of handling using telemetry to study the blood pressure changes during the time when animals are handled and all the subsequent stages of the tail-cuff protocol.
- To investigate the impact of the handler's gender on the blood pressure and heart rate of the mouse during the handling and the subsequent stages of the tail-cuff protocol.

3.2. Protocol details including experimental design and development

For detailed methods, including the different handling techniques, please refer to Chapter 2.

3.2.1. Animals

Male CD1 (n=15) and C57Bl6/J male (different groups of a total of 22 mice) and C57Bl6/J female (n=7) mice were included in the studies to characterise the tail-cuff protocol and the handling studies that only used the tail-cuff technique. Male CD1 mice were bought from Charles Rivers (UK) and were allowed to acclimatise in the animal holding facility for one week. The age range of the CD1 mice used in these experiments was 12 -13 weeks. All C57Bl6/J mice used in these experiments were bred in house and the age range of the mice was 12-14 weeks at the start of the experiments. An additional 6 male C57Bl/6J mice were used in the further telemetry-based experiments (3 of which were further used in the study that explored the effect of handler's gender on the mouse's blood pressure during handling and in the context of the tail-cuff protocol). The age range of the mice used in these experiments at the start of the procedures was 15 - 16 weeks.

3.2.2. Measuring blood pressure

3.2.2.1. Tail-cuff plethysmography

The initial phase of the project was to characterise the tail-cuff technique to record blood pressure in mice. The tail-cuff protocol previously used in the laboratory, in conjunction with the manufacturer's instructions, were used as a guidance in planning the studies for the initial stage of the project. Male CD1 (n=15) and C57Bl6/J (n=7) mice were used at this stage of the project. The tail-cuff protocol was as described in section 2.4.1. The mice were trained or acclimatised to the tail-cuff recording sessions over 14 sessions on 14 consecutive days. Following this period, the mice were considered to be ready for the recording of "baseline" blood pressure.

3.2.3. Handling protocol for the tail-cuff only study.

C57Bl/J male (n=5) and female (n=7) were habituated to the tail-cuff technique and handling on at least 5 occasions on 5 consecutive days. The capture method at the training stage was usually the tail-cup technique because I found it easiest to use in the naïve mice. Following a rest period of 5 days, the mice were assigned to a specific handling technique and were captured using this technique to measure their blood pressure by tail-cuff on 4 to 5 consecutive days. Following a rest period of 4 to 5 days, the mice were handled by another technique to have their blood pressure measured by the tail-cuff. After the three cycles of tests, each mouse was subjected to: tube, hand-cup, or tail-cup handling technique before their blood pressure was measured by the tail-cuff system. The "hand-cupping" technique was not always reproducible in all mice and was more challenging to use to capture mice not used to handling in general; therefore this technique was not further explored in the experiments that followed.

The following study was designed to compare two handling techniques. I continued using cross-over design for the subsequent experiments. Eight male C57Bl6 mice, who were housed in 2 separate cages of 4 mice in each cage, were split into two groups: one group (cage) was handled using the home tunnel (the "tube"), for 4 days, while the other group (cage) of mice were handled by the tail. Following three days of rest, the handling techniques were switched between the groups of mice: the mice that were handle by the home tunnel were handled by the tail for 4 days and vice versa. All handling of mice within the period of the study was carried out by myself using either the tube or tail capture techniques as per the group allocation. Before the study commenced, I most often used the handling technique "tail-cup", due to convenience. The number of faecal pellets each mouse deposited during the tail-cuff protocol was used as a simple marker of anxiety. It is noted that collecting this data did not cause any further distress to the mouse.

3.2.4. Protocol for the handling for the telemetry and the tail-cuff study

Male C57Bl6/J mice (n=6) were habituated to the tail-cuff protocol on at least 7 occasions before being implanted with blood pressure radiotelemetry devices. Following at least 10 days of recovery period, blood pressure, heart rate and activity were recorded over at least 24 hours to establish baseline parameters and confirm successful recovery from the surgery. Telemetry was used to monitor blood pressure and heart rate during the length of the following experiment.

The mice (n=6 in total) were randomised to handling sequences (n=2 per sequence) as shown in figure 3.1. For this study the following handling techniques were used: tube, tail, tail-cup (as described in Chapter 2 section 6), followed by measurement of blood pressure (chapter 2.4.3).

Each mouse was handled by each handling technique for the purposes of the tail-cuff protocol and other routine procedures such as cage cleaning and weighing on 5 consecutive days. The handling periods were separated by six days of rest, when handling was restricted to welfare checks when deemed necessary.

Mice trained to the tail-cuff technique	Telemetry surgery + 10 day recovery	Animal groups	Handling Week 1	Rest, 6 days	Handling Week 2	Rest, 6 days	Handling Week 3
		ABC	A		B		C
		BCA	B		C		A
		CAB	C		A		B

Figure 3.1. Schematic of the experimental design for the study of the effect of the handling techniques on blood pressure and heart rate. Animal groups represent different sequence of the handling techniques used. Each mouse was handled by each of the handling techniques tested in the sequence shown: A=tube handling, B=tail-cup handling, C=tail handling. Six mice were used in this study.

The tail-cuff protocol was arbitrarily subdivided into the following sequential steps:

- "baseline" (the period before the animals were handled), typically a 15-minute period when the mouse was quiet before handling or moving the case
- handling (typically 10-30sec) – the period during which the mouse was captured and lifted out of the home cage using a specified handling method ready to be placed into the tail-cuff holding tube
- placing the animal in the restraint tube (typically 30-60sec) – the mouse is encouraged to walk into the tail-cuff holding tube

- restraint in the tube - acclimatisation in the tail-cuff holding tube (typically 5 min), which is maintained at 33-35°C at the heating platform

- tail-cuff recording – repeated tail-cuff inflation and deflation cycles (2 cycles per minute). Each recording session would typically consist of 20-25 measurements lasting 10-13 minutes.

Following the completion of the procedure, the mouse is returned to the home cage for further monitoring.

3.2.5. The effect of handler's gender on the mouse blood pressure and heart rate during the tail-cuff protocol

Tail-cuff protocols and telemetry recordings were carried out as described above. Each researcher handled 3 mice by their preferred technique (techniques used were recorded). Male (n=3) and female (n=4) researchers alternated to carry out the measurements on consecutive days.

3.2.6. Statistical analysis

Sample size calculations were carried out based on the assumptions and criteria as described in section 2.9. Initially, standard deviation and the difference between the means were estimated from previous work in this laboratory (Smillie et al, 2014) and were as follows: σ=10 (mmHg), $\mu 2$-$\mu 1$ =10 (mmHg). According to the assumptions used, the required sample size was n=8 per study group. Since the potential effect of the different handling techniques on the blood pressure was not known, including the effect of handling and other interventions on central blood pressure, smaller pilot study will be run and the number of animals will be increased as necessary. This approach will be adopted in all the telemetry studies.

Statistical analysis was performed using GraphPad Prism 5.0 in all cases apart from figure 3.12, in which case IBM SPSS Statistics 22 software was used to perform a more advanced ANOVA for Latin Square design. Data in this chapter of the manuscript is typically expressed as mean ± SEM. The mean value was typically calculated for each group either for either for each session or over the treatment period as applicable. Further detail regarding the statistical analysis is provided in each figure.

3.3. Results

3.3.1. Characterisation of the tail-cuff protocol

The immediate problem that I encountered using the tail-cuff system for the first time, as an inexperienced operator was that it was difficult to obtain a sufficient number of software-accepted blood pressure measurements. Moreover, many of the software-accepted readings were not comparable to the published results that also used the same technique. Heating and restraint were sought to be optimised in the first instance to address the problem of high rejection rate for the measurement cycles.

I explored if there is a relationship between the achieved tail volume and the measured systolic blood pressure in the tail. Figure 3.2 shows the result of this analysis. There appears to be a non-zero order relationship between the systolic pressure and volume measurements below the volume of 15 µl for most measurement cycles. Above the 15 µl volume, there is no apparent trend for the systolic pressure to increase or decrease with increasing volume. Considering the fact that the VPR system relies on the volume change in the tail during the reperfusion; the results indicate that the measurement cycles that did not achieve at least 15 µl volume increase (as also illustrated by figures 2.3 A and B) are not reliable. I believe that this observation further supports the manufacturer's recommendations to use this volume as a lower cut-off criterion for accepting the readings.

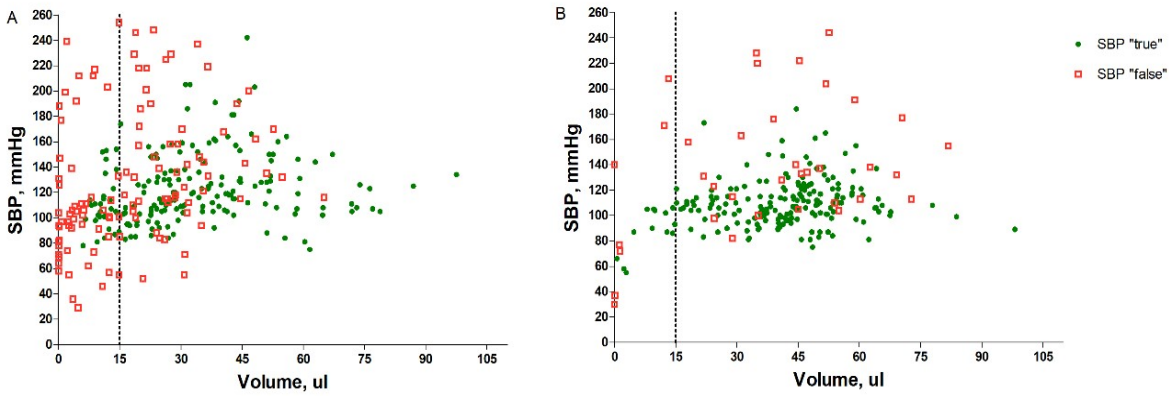


Figure 3.2. Correlation between the tail volume and systolic blood pressure (SBP) over a recording session. *(A) CD1 male mice, n=15, (B) C57Bl6/J male mice, n=5, showing "true" (software-accepted), and "false" (software-rejected) measurements.*

The problem of obtaining sufficient number of valid measurements was particularly acute at the very beginning when I first started using the tail-cuff technique, and it persisted more with the CD1 strain of mice. Insufficient blood volume in the tail appeared to be more common in the CD1 strain. Figure 3.2A shows that there are more cycles that achieved less than 15 µl increase in the tail volume in CD1 mice compared to the C57Bl6/J strain. Based on this observation, this factor

was thought to be at least in part responsible for higher rejection rate of the measurement cycles with CD1 mice. The majority of the software-rejected cycles for C57Bl6/J (figure 3.2B), as well as a significant proportion of the rejected cycles with the CD1 mice (figure 3.2A), were most likely due to movement artefacts.

C57Bl6/J mice were included into the study within a week of initiating the recordings with the CD1 mice. It was my experience that the C57Bl6 strain used in this study appeared more amenable to the tail-cuff protocol for the following reasons: 1) they appeared calmer during the handling and moved less during the recording sessions, 2) the recording sessions with this strain yielded a higher proportion of "true" readings by the 12^{th} recording session. Figure 3.3A shows the blood pressure profiles for the two strains of mice that were used at the start of the study (both performed by "inexperienced operator") and blood pressure measurements obtained approximately 5 months later using a different group of C57Bl6 strain (termed as "experienced operator"). The data shown are following 5 days of "training" or "acclimatising", i.e. the period that was still considered as the "training" period according to the original protocol. Several points can be noted: 1) there is no trend for the blood pressure measurements to decrease (or increase) as the recording sessions progressed, 2) both strains used at the start of the project had similar blood pressures 3) that was overall similar to the readings obtained later in the project with C57Bl6 mice.

Although both strains yielded comparable blood pressure readings (figure 3.3A), the recording sessions with the C57Bl6 mice resulted in more "true" readings over time (figure 3.3B). Following 12 "training" sessions, an "inexperienced" operator could obtain approximately 70% of true readings with C57Bl6 mice, while the "true" readings for CD1 mice remained low at approximately 30%. The sessions run by the "experienced" operator yielded significantly higher proportion of "true" measurements from the 6^{th} recording session that was maintained throughout 80% throughout (figure 3.3B).

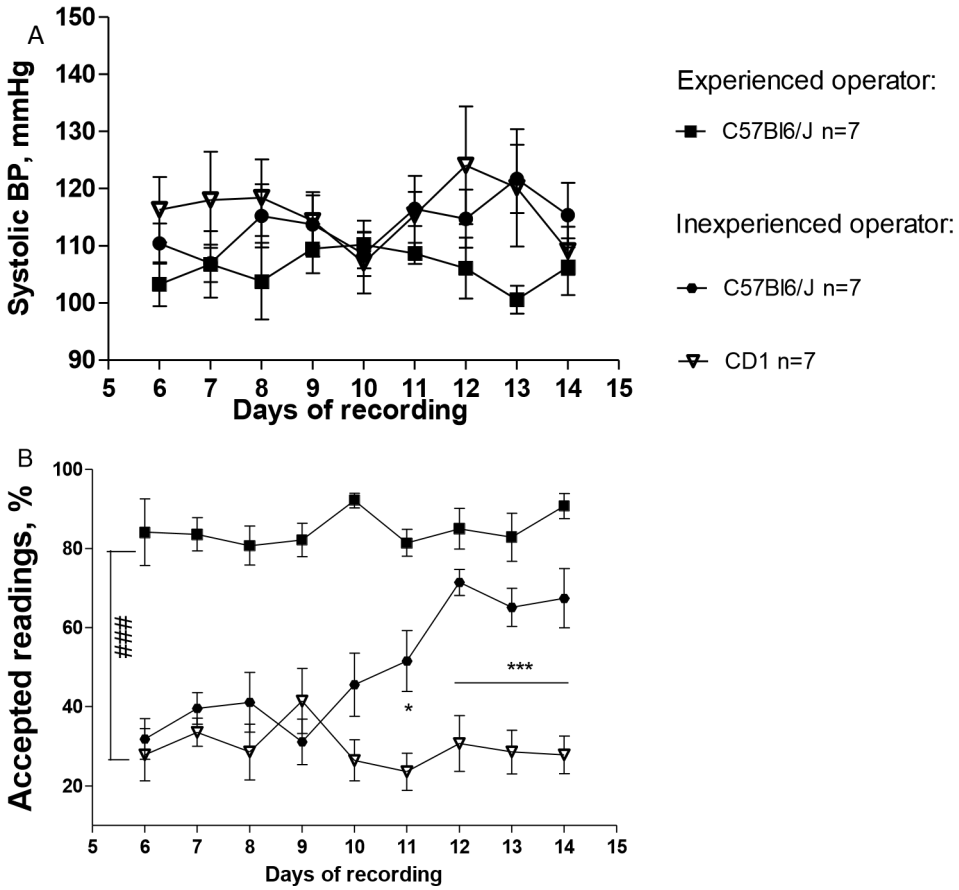


Figure 3.3. Effect of operator training on systolic blood pressure (A) and the number of accepted readings (B). *A) Systolic blood pressure (SBP) measurements in male CD1 (n=7) and male C57Bl6/J (n=7) mice performed by inexperienced operator and SBP recordings in male a different group of male C57Bl6/J mice (n=7) performed by the same operator once she obtained more experience in the technique. B) The number of the accepted readings for each of the corresponding days of the recording as shown in (A) In B; significant differences are shown between the CD1 and C57Bl6/J mouse groups (*p<0.05 for day 11, ***p<0.001 thereafter) when an expert is compared with inexperienced operator. The number of accepted readings obtained by the experienced operator was significantly higher (### p<0.001) throughout compared to the number of readings obtained by the inexperienced operator for CD1 mice. Two-way ANOVA and Bonferroni post-hoc comparison was performed using GraphPad software.*

3.3.2. Characterising the effect of different handling techniques on blood pressure measurements by the tail-cuff

Male and female C57Bl6/J mice were trained for the tail-cuff protocol on at least 5 occasions and their blood pressure profiles are shown in figure 3.4. No significant difference in blood pressure is found between males and females, despite variability in blood pressure within each group.

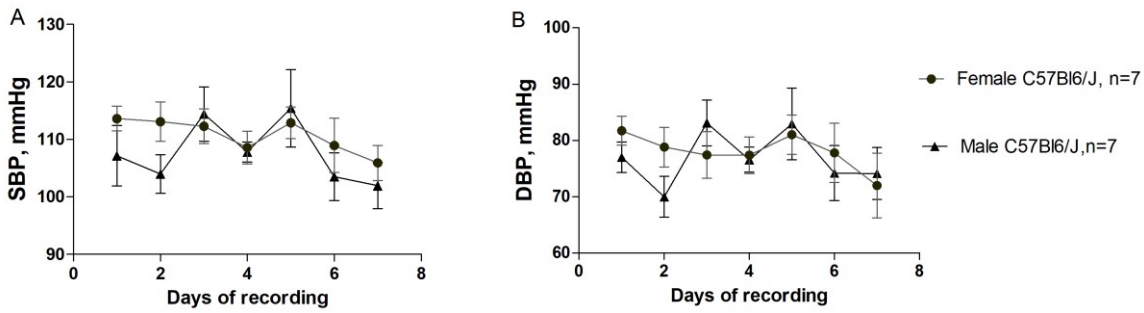


Figure 3.4. Blood pressure measurements for male and female C57Bl6/J mice as measured by the tail-cuff. Measurement after the mice were habituated to the protocol on at least 3 occasions. (A) SBP – systolic blood pressure and (B) DBP - diastolic blood pressure. Each data point represents mean ± SEM for males (n=7) and female (n=7) mice. No significant difference was found at the 5% significance level using 2-way ANOVA.

3.3.2.1. The effect of different handling techniques on blood pressure of mice as determined by the tail-cuff system

Male and female C57Bl6/J mice were repeatedly handled by each of the defined handling techniques and subjected to the tail-cuff protocol. The handling techniques were: tube, tail-cup and hand-cup, according to the experimental design section. The resultant blood pressure measurements (figure 3.5) were similar for all the handling techniques. Females were less amenable to capture by the hand-cup technique and only four out of seven female mice could be caught in the cup of the hand without apparently distressing the animals. The male mice in this cohort had similar responses, in terms of blood pressure measurements by the tail-cuff technique and the observed response to capture by each defined technique.

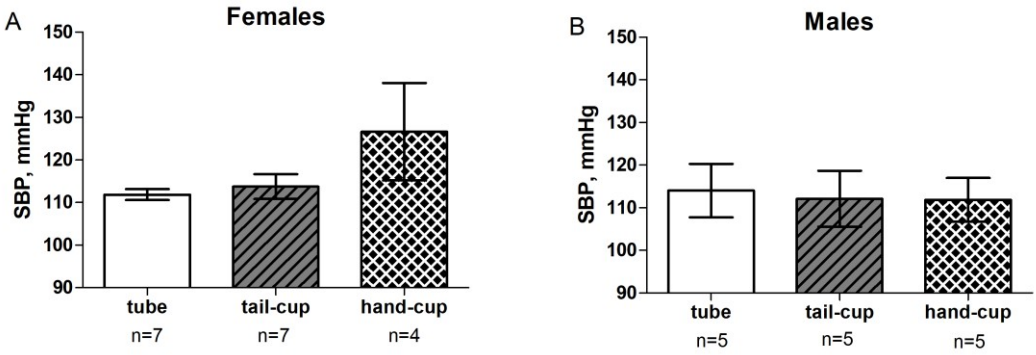


Figure 3.5. The effect of handling by tube, tail-cup or hand-cup on A) Systolic blood pressure (SBP) of A) female and B) male C57Bl6/J mice as determined by the tail-cuff technique. Each data point represents mean ± SEM for males (n=5) and female (n=7 or n=4) mice. No significant difference was found at the 5% significance level using one-way ANOVA.

The "tail-cupping" technique described by Hurst and West (2010) appeared to cause more distress in some mice and it was difficult to catch some mice in this way without causing them apparent distress. Therefore, I went on to exclude "hand-cup" and compared the two techniques: using the tube and the tail as described by Hurst and West (2010), that were in most contrast to

each other. Hurst and West (2010) reported that CD1 and C57Bl6 mice handled by tube were less anxious than their tail-handled counterparts. I tested only acute effects on blood pressure during the tail-cuff protocol in C57Bl6/J mice. My end-point measurements were blood pressure (as determined by the tail-cuff technique) and the number of faecal pellets; as show in figures 3.6 and 3.7.

Figure 3.6 A-C demonstrates that that both techniques that were used to capture the mice to undergo the tail-cuff protocol produced similar blood pressure measurements by the tail-cuff system. The number of faecal pellets was also similar. Although it was not officially measured, both capture techniques were similar in the amount of time it took to capture the mice and then place a mouse into the restraint tube. However, some mice were easier to catch with a tunnel and some were by the tail.

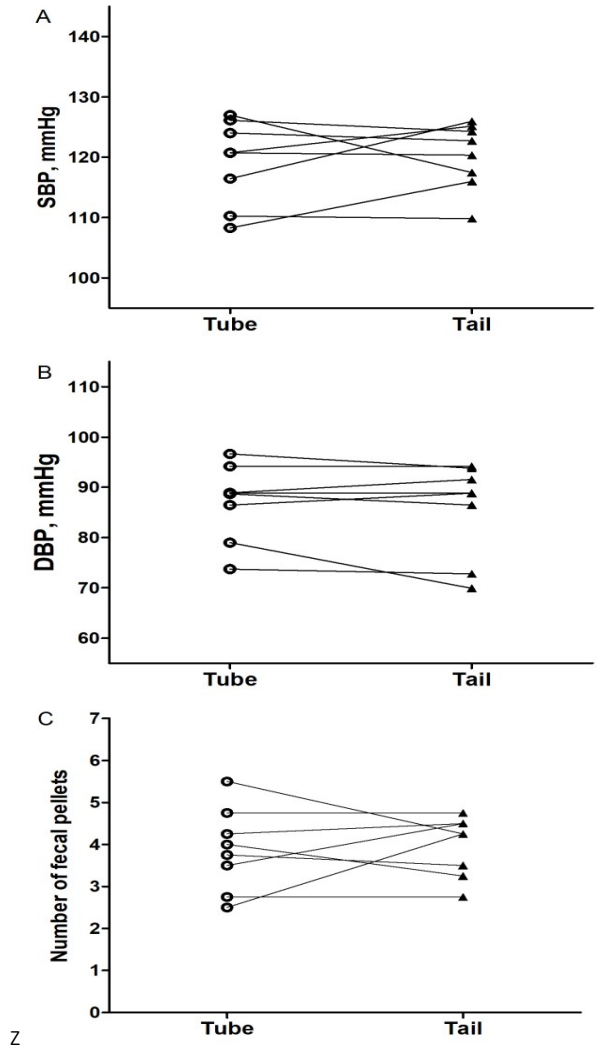


Figure 3.6. The effect of handling by tube and the tail on (A) systolic and (B) diastolic blood pressure, and (C) the number of faecal pellets deposited by each mouse during the tail-cuff protocol. *Male C57Bl6 mice (n=8) were captured by each of the two handling technique and blood pressure measured by the tail-cuff protocol (A-B). In the same experiments, the number of faecal pellets (C) was counted. Each data point represents an average value for one mouse over a period of four days of measurements for each respective technique.*

Having not observed any difference in the blood pressure recordings by the tail-cuff system or the number of pellets deposited by the mice handled by these two handling techniques, I used the data generated in these experiments to explore if there was a trend in terms of lower blood pressure or lower variability of the measurements, as well as fewer faecal pellets deposited during the tail-cuff protocol by arranging the data in chronological order (figure 3.7). There is no overall trend for blood pressure or its variability (seen by the similar error bars, figure 3.7A), or the number of faecal pellets (figure 3.7B) to change over time. I interpret these results as evidence that the mice are not becoming more accustomed to the tail-cuff protocol.

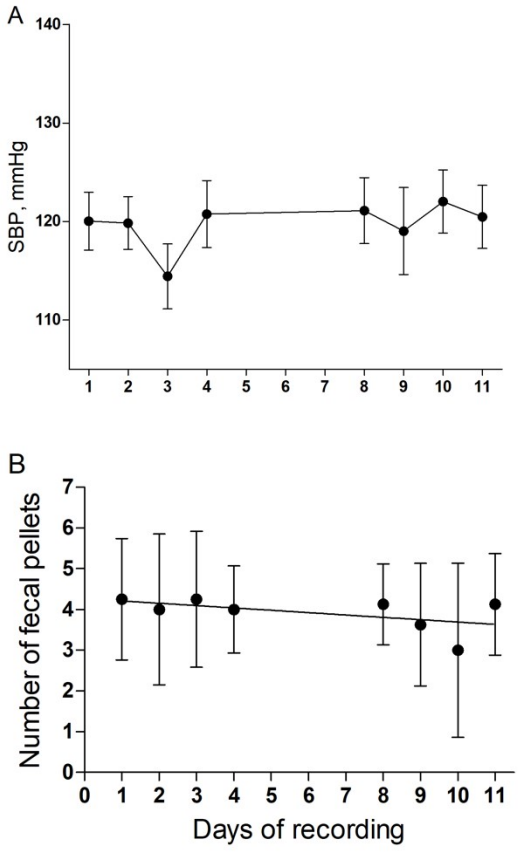


Figure 3.7. The effect of progressive recording (in chronological order) by the tail-cuff technique on (A) the systolic blood pressure (SBP) and (B) the number of faecal pellets. Each data point represents the mean ± SEM for 8 male C57Bl6/J mice on each day of the recording. Solid line in (B) represents the regression line ($p=0.216$, $r^2=0.02029$).

3.3.3. Telemetry- based study to assess the effect of different handling techniques on acute blood pressure during the tail-cuff protocol.

When carrying out the previous study, I realised that mice appeared stressed, during the tail-cuff technique but had systolic blood pressure readings of about 100 – 130 mmHg and diastolic pressure of 65 – 90 mmHg, not in keeping with that expected in a stressed mouse. The VPR tail-cuff system that I used during this project did not give a reliable estimate of heart rate. Also, the tail-cuff technique does not allow blood pressure to be measured during or before the handling

technique. Telemetry, by comparison, allows high resolution recording of blood pressure and heart rate in the conscious mouse and its use was included in the experiments described below.

To investigate the impact of each defined handling technique on blood pressure and heart rate during handling and the other stages of the tail-cuff protocol, the tail-cuff protocol has been subdivided into the following steps: 1) removing the cage lid, 1) picking up the mouse (handling), 2) placing the mouse into the tail-cuff holding tube, 3) acclimatisation – restraint and warming to 33-35°C in the tail-cuff holding tube, 4) tail-cuff recording – 20-25 cycles of tail-cuff inflations and deflations. These steps were identified as the basic identifiable units of intervention.

Results are shown for the following three different handling techniques as described in the methods section in more detail; namely tube; tail-cup and tail:

The "hand-cupping" technique described by Hurst and West (2010), appeared; as described above, to cause distress. Therefore the "cup" technique was replaced with the "tail-cup" technique that I was familiar with. Although the handling by the tail as described by Hurst and West (2010) was avoided where possible, this technique was used in this study because it remains a widely used technique. Considering possible pro-anxiogenic effects in mice, it is important to learn if there is an effect on cardiovascular system particular to this technique.

As in the previous handling study, each mouse underwent each handling technique separated by a washout period of at least 6 days. The time for starting each defined step ("handling", "placing into the tube", "restraint", "tail-cuff inflations") was noted, usually within the precision of 5 seconds on average. Clocks of the tail-cuff and telemetry computers were synchronised on each day and both, or the tail-cuff computer clock was used for timekeeping.

3.3.3.1. Tube

Figure 3.8 shows the mean ±SEM values for each of the steps, starting with baseline, for the mice handled using the tube. Due to the equipment arrangement constraints during a part of this series of experiments, the telemetry recording after the tail-cuff procedure was complete, was prone to be affected by other variables, such as cages that had to be moved. Thus' the period for recording was limited and it was decided to measure and report the cardiovascular parameters, through use of telemetry, for a 15 minute time point "15 min after" after the tail-cuff procedure was complete and the animals were returned to their home cages. Baseline is typically a period before the animal is disturbed in any way and is recorded while the animal is in its home cage. The "15 min after" period is also recorded when the animal is in the home cage. Handling usually

started when the animal is captured in the home cage and is transferred to the tail-cuff platform, where the “placement into tube”, “Restraint” and “Cuff inflations” take place.

Significant increases in blood pressure and heart rate (###, $p<0.001$) are observed for the mice during handling using the tube. The mean increase in blood pressure was 26.9 mmHg (95% CI 10.7 to 43.1) for SBP, 19.1 mmHg (95% CI 6.7 to 31.3) for DBP and 243.4 beats per minute (95% CI 173.3 to 295.2) for heart rate. There is a trend for blood pressure, and also marginally for the heart rate, to increase further reaching the maximum during the restraint period. This increase reaches significant level (*, $p<0.05$) compared to the handling period for the diastolic pressure, but not the other measured parameters. Although the blood pressure or heart rate were not further elevated when the occlusion cuff over the mouse’s tail started to inflate, there was no significant decrease during the tail-cuff recording period either. The mice were returned to the home cages using the same handling technique they are assigned to. Fifteen minutes after the mice were returned to their home cages following the tail-cuff protocol, they still had elevated blood pressure (#, $p<0.05$) and heart rate (###, $p<0.001$) compared to the baseline.

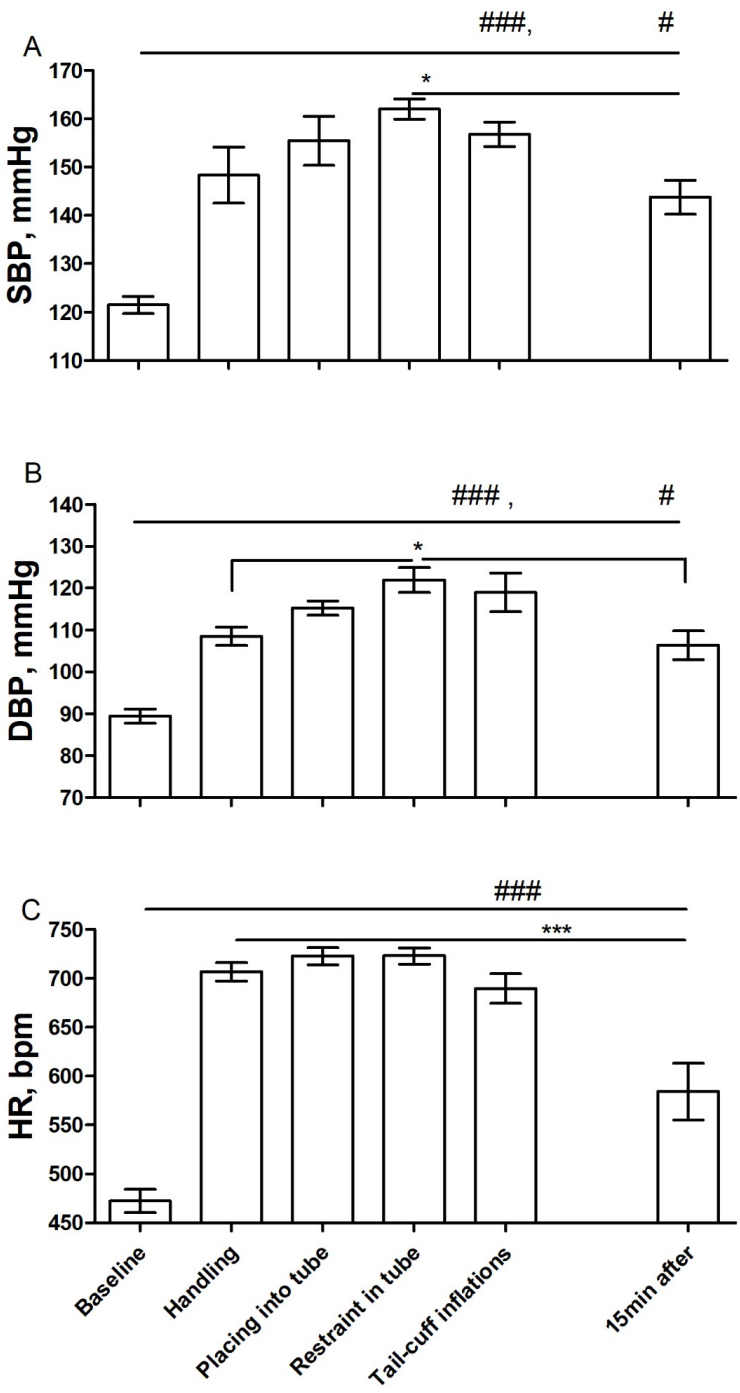


Figure 3.8. Tube handling – systolic and diastolic blood pressure (A, B) and heart rate (C) values during the defined stages of the tail-cuff protocol as measured by telemetry. *Each stage of the protocol is significantly different from baseline (###, p<0.001); the measurement taken 15 minutes after return to home cage is also different from the baseline (*, p<0.05) using ANOVA and Tukey's Multiple Comparisons test with Bartlett's test for sphericity. Data shown as mean ± SEM for 6 male C57Bl6/J mice over 5 test sessions when the animals are handled by tube.*

3.3.3.2. Tail-cup.

A similar pattern of changes of blood pressure and heart rate occur when the animals are handled using the tail-cup technique (figure 3.9). Systolic pressure and heart rate rose significantly upon

handling (###, p<0.001), with insignificant further changes during the placement into the tube and restraint. The mean increase compared to baseline was 29.1 mmHg

(95% CI 13.4 to 44.8) for SBP and 226.4 (95% CI 172.4 to 280.3) beats per minute for heart rate. Systolic pressure remained at the same high level throughout the recording by the tail-cuff system (###, p<0.001 relative to baseline). Both systolic blood pressure and heart rate were also significantly different from the measurements 15 minutes after the mice were returned to the home cage (##, p<0.01). Interestingly, changes in diastolic pressure during the tail-cup handling were not significantly different from baseline (mean difference 22.2 mmHg, 95% confidence interval -6.17 to 50.5), nor placing into the tube (mean difference 27.6 mmHg). The difference became significant during the restraint (#, p<0.05, mean difference 32.8 mmHg) and tail-cuff inflations (##, p<0.01, mean difference 34.3 mmHg).

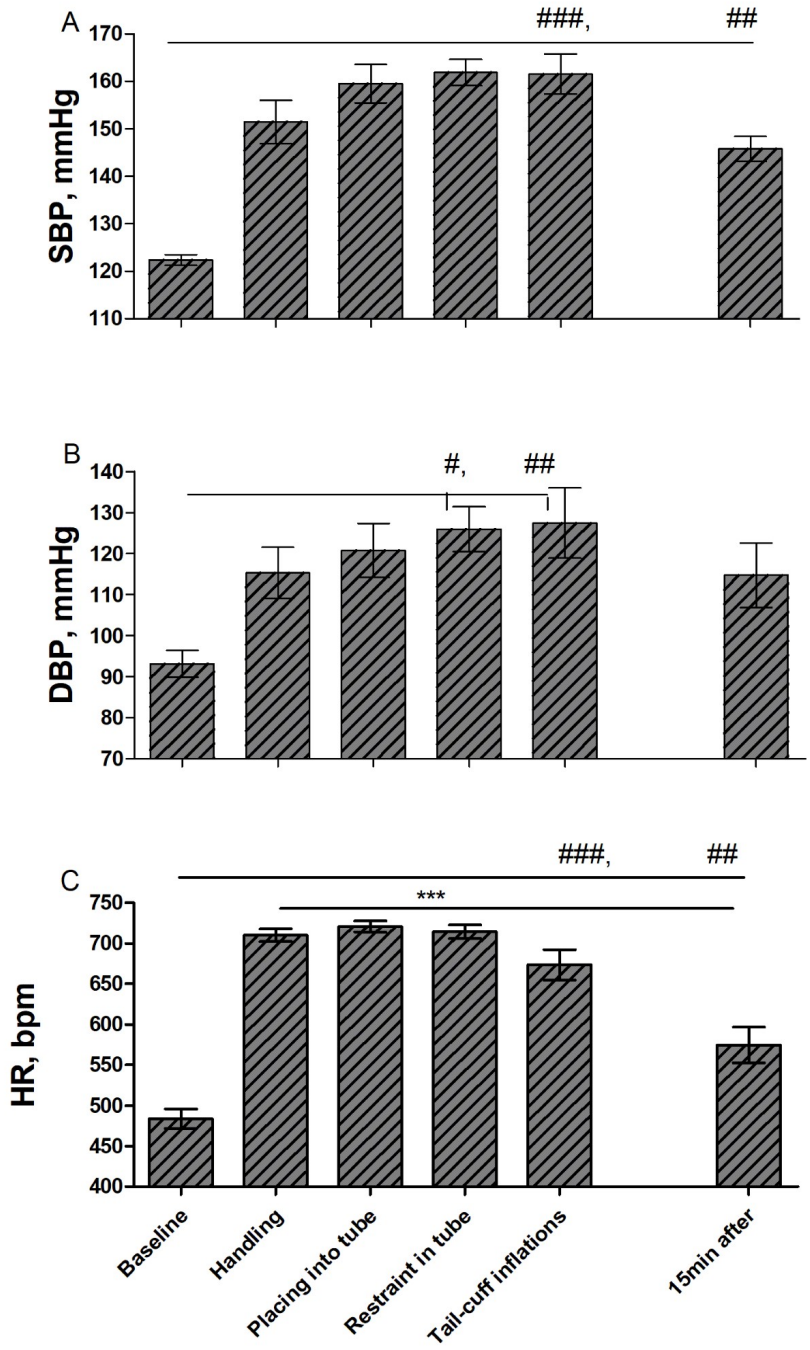


Figure 3.9. Tail-cup handling – systolic and diastolic blood pressure (A, B) and heart rate (C) values during the defined stages of the tail-cuff protocol as measured by telemetry. *Each stage of the protocol is significantly different from baseline (###, p<0.001); 15 minutes after time point is also different from the baseline (*, p<0.05) using ANOVA and Tukey's Multiple Comparisons test with Bartlett's test for sphericity. Data shown as mean ± SEM for 6 male mice over 5 test sessions when the animals are handled by tail-cup.*

3.3.3.3. Tail-handling

I was surprised to discover that the final handling technique, tail handling induced similar increases in blood pressure and heart rate during the handling process itself (figure 3.10): mean increase of SBP compared to baseline reached 25.9 (95% CI 10.1 to 41.3, ### p<0.001) mmHg, 19.6 (95% CI 0.4 to 39.7) mmHg for DBP, although the latter change was not significant; mean

increase in heart rate was 224.7 (95% CI 169.0 to 280.8). The subsequent changes in cardiovascular parameters during the tail-cuff protocol for the tail-handled mice also progressed in a similar manner. Systolic blood pressure remained significantly elevated throughout the protocol, with the mean increase from baseline reaching the maximum value of 39.4 mmHg (95% CI 23.4 to 55.3) during the cuff inflations. Diastolic pressure also reached maximum increase relative to baseline during the recording period by the tail-cuff system, mean difference 34.4 (95% CI 13.4 to 54.5, ###, p<0.001) mmHg.

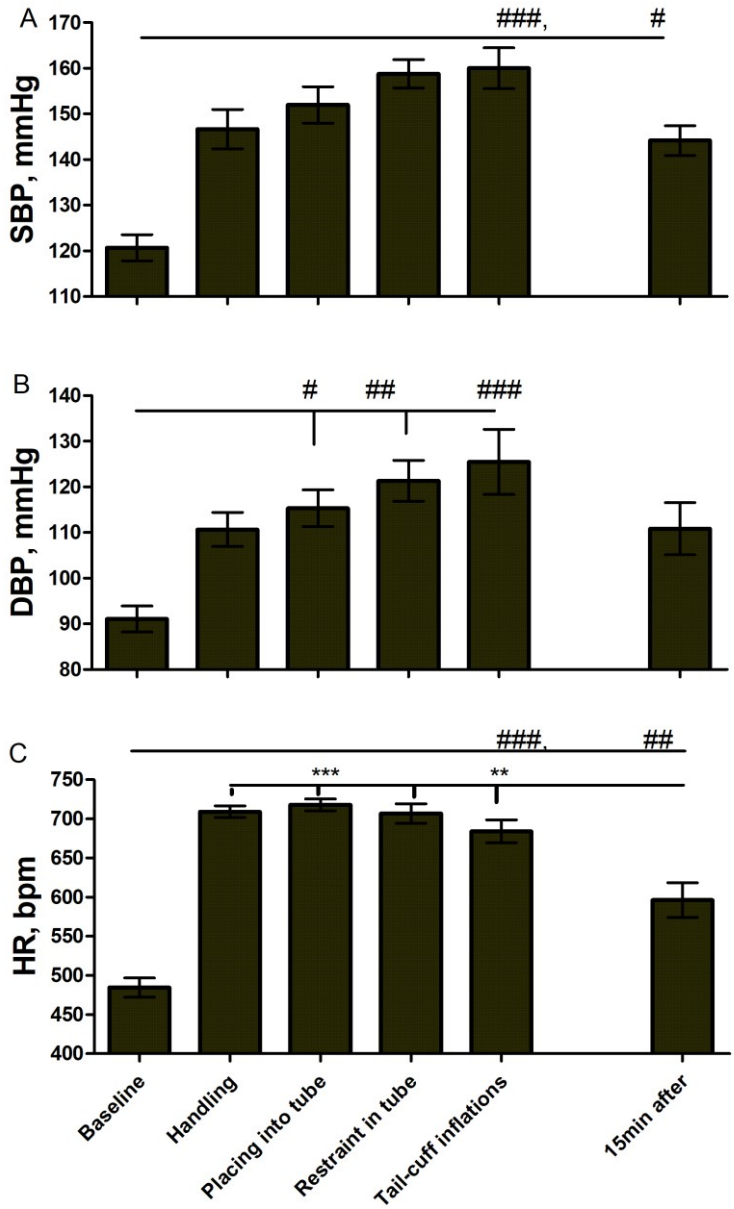


Figure 3.10. Tail handling – average blood pressure (A, B) and heart rate (C) values during the defined stages of the tail-cuff protocol as measured by telemetry. *Each stage of the protocol is significantly different from baseline (###, p<0.001); 15 minutes after time point is also different from the baseline (*, p<0.05) using ANOVA and Tukey's Multiple Comparisons test with Bartlett's test for sphericity. Data shown as mean ± SEM for 6 male mice over 5 test sessions when the animals are handled by the tail.*

When the three handling techniques were compared against each other using ANOVA for Latin Square design (SPSS22 software), they did not differ in the manner that the mouse's blood pressure and heart rate (figure 3.11) were affected during the handling or later stages of the tail-cuff protocol. The sequence in which the handling techniques were tested in mice was shown to have no effect on the results. Similar large increases in blood pressure and heart rate compared to baseline were observed for all the techniques during the handling ($p<0.001$), which tended to peak during the restraint (usually not significantly different from the handling period) and were maintained throughout the protocol and at least up to 15 minutes after when the mice were returned to their home cages ($p<0.001$).

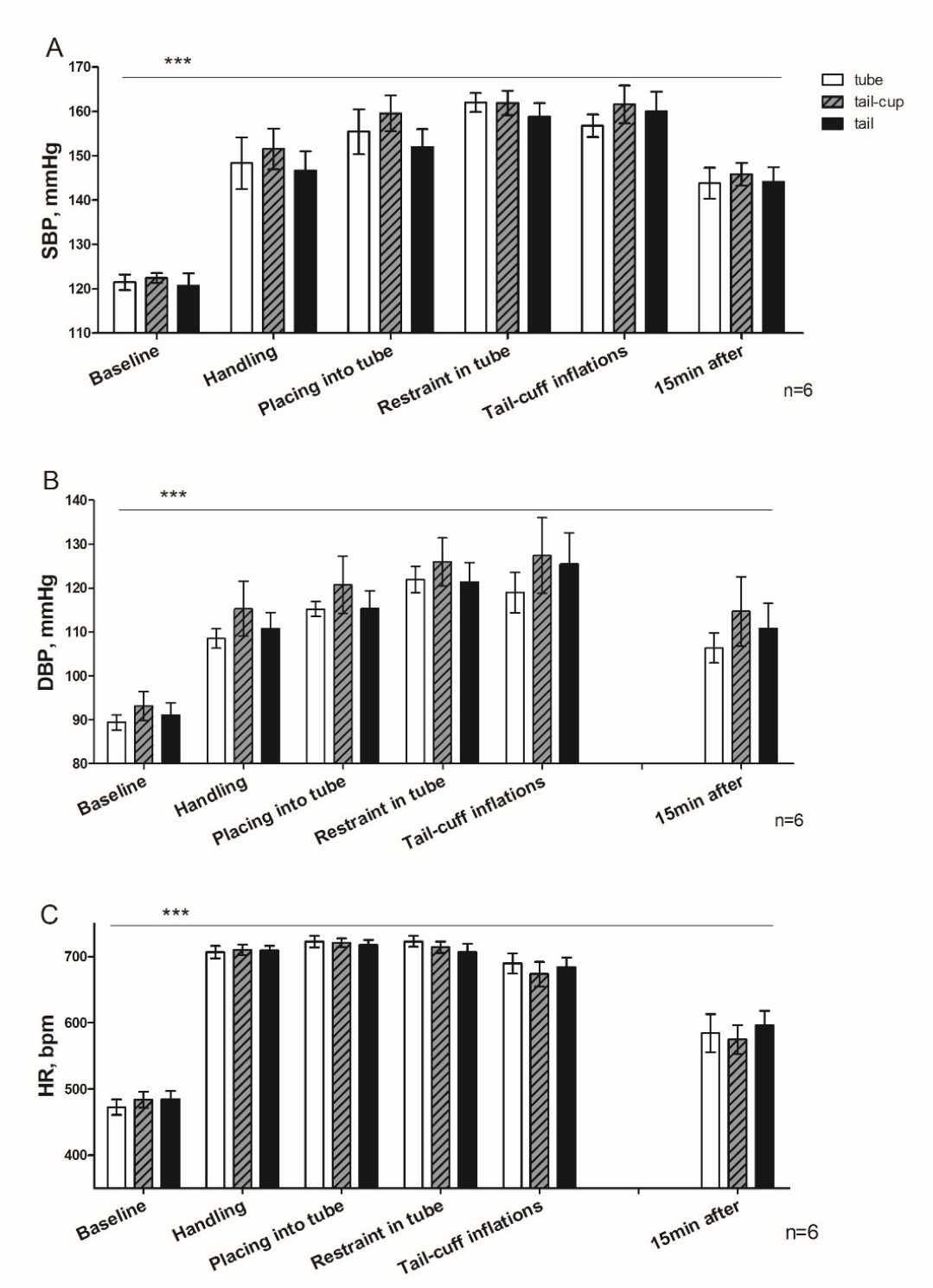


Figure 3.11. A summary graph to show the comparative data from the different handling techniques used (tube, tail-cup and the tail). *Effect on systolic blood pressure (A), diastolic blood pressure (B) and heart rate (C) during the various stages of the tail-cuff protocol as measured by telemetry. There is no significant difference between handling techniques at any stage of the protocol (p>0.05), however each stage of the protocol is significantly different from baseline apart from the 60 min time point. Data shown as mean ± SEM for 6 male mice over 5 test sessions. ANOVA for Latin-Square design analysis was performed using SPSS22 software.*

3.3.3.4. Longitudinal analysis of blood pressure changes during tail-cuff and effect of moving cage lid.

The stages of the tail-cuff protocol were further scrutinised to include the very first step, the removal of the cage lid by the investigator. Instead of the average values for each period as used in the figures 3.8-3.11), the handling phases were aligned as 10 second intervals. The graphs below (Figures 3.12-3.15) all show a similar trend where the mode of handling does not appear to affect the final results but that lifting the cage lid is the initial stimulus for increasing the heart rate especially, as measured by telemetry.

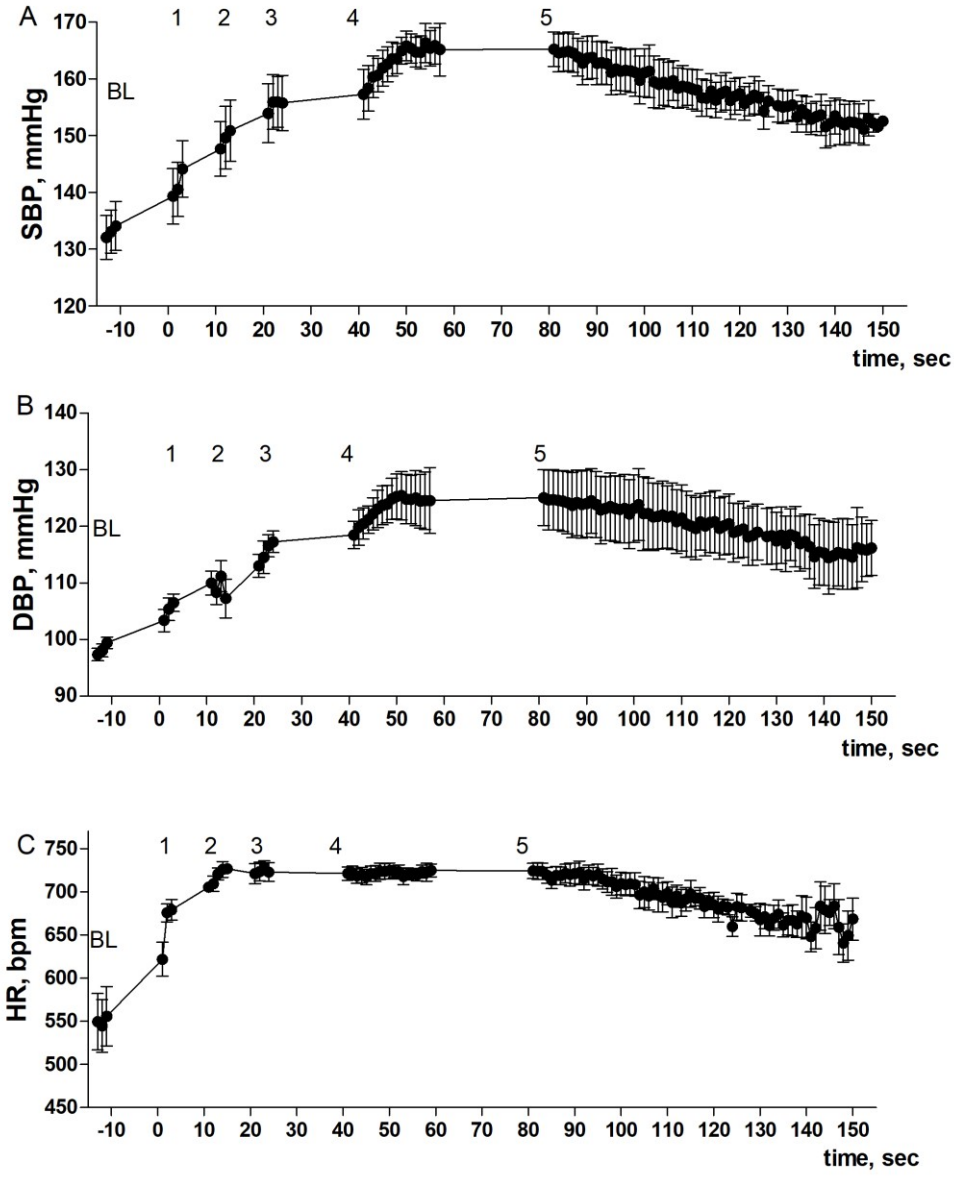


Figure 3.12. Tube handling. Intervention phases of the tail-cuff protocol are displayed longitudinally at the temporal resolution of 10 seconds. Each data point is a 10 second average (±SEM) for 6 mice over 5 recording sessions when handled by the tube. Time "0" is when the cage lid was lifted. BL = baseline, 1 = cage lid is lifted, 2 = handling, 3 = placing into the tail-cuff holding tube, 4 = acclimatisation in the tail-cuff holding tube, 5 = inflation cycles of the cuff over the mouse's tail.

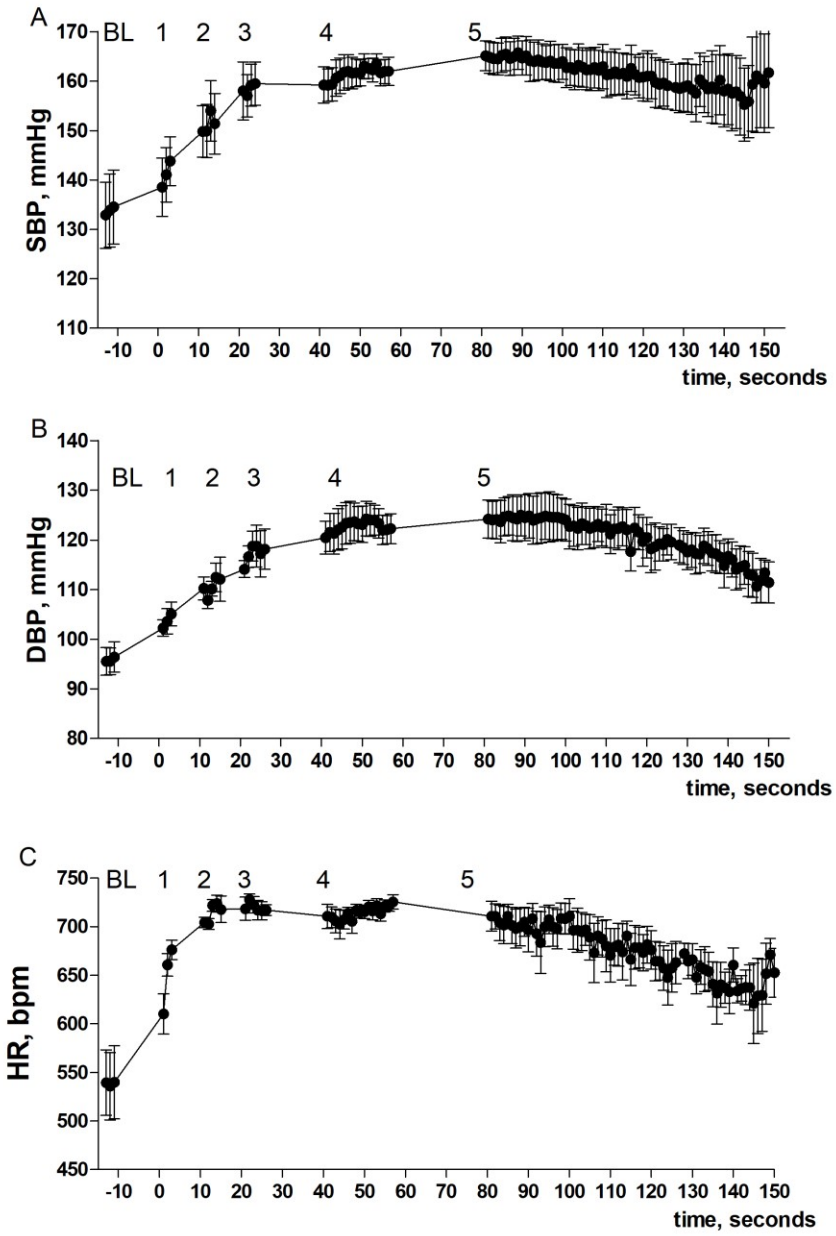


Figure 3.13. Tail-cup handling. Intervention phases of the tail-cuff protocol are displayed longitudinally at the temporal resolution of 10 seconds. Each data point is a 10 second average (±SEM) for 6 mice over 5 recording sessions when handled by the tail-cup technique. Time "0" is when the cage lid was lifted. BL = baseline, 1 = cage lid is lifted, 2 = handling, 3 = placing into the tail-cuff holding tube, 4 = acclimatisation in the tail-cuff holding tube, 5 = inflation cycles of the cuff over the mouse's tail.

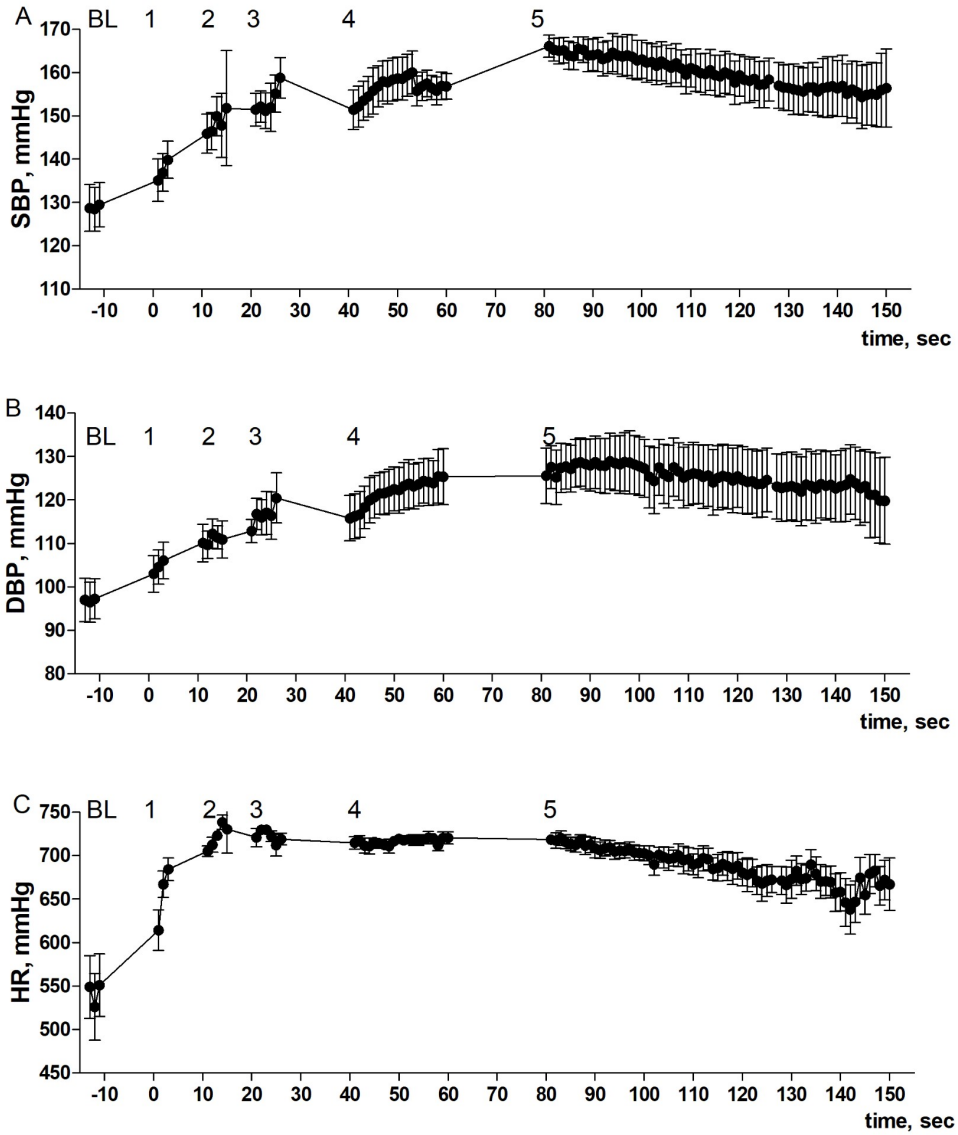


Figure 3.14. Tail handling. Intervention phases of the tail-cuff protocol are displayed longitudinally at the temporal resolution of 10 seconds. *Each data point is a 10 second average (±SEM) for 6 mice over 5 recording sessions when handled by the tail. Time "0" is when the cage lid was lifted. BL = baseline, 1 = cage lid is lifted, 2 = handling, 3 = placing into the tail-cuff holding tube, 4 = acclimatisation in the tail-cuff holding tube, 5 = inflation cycles of the cuff over the mouse's tail.*

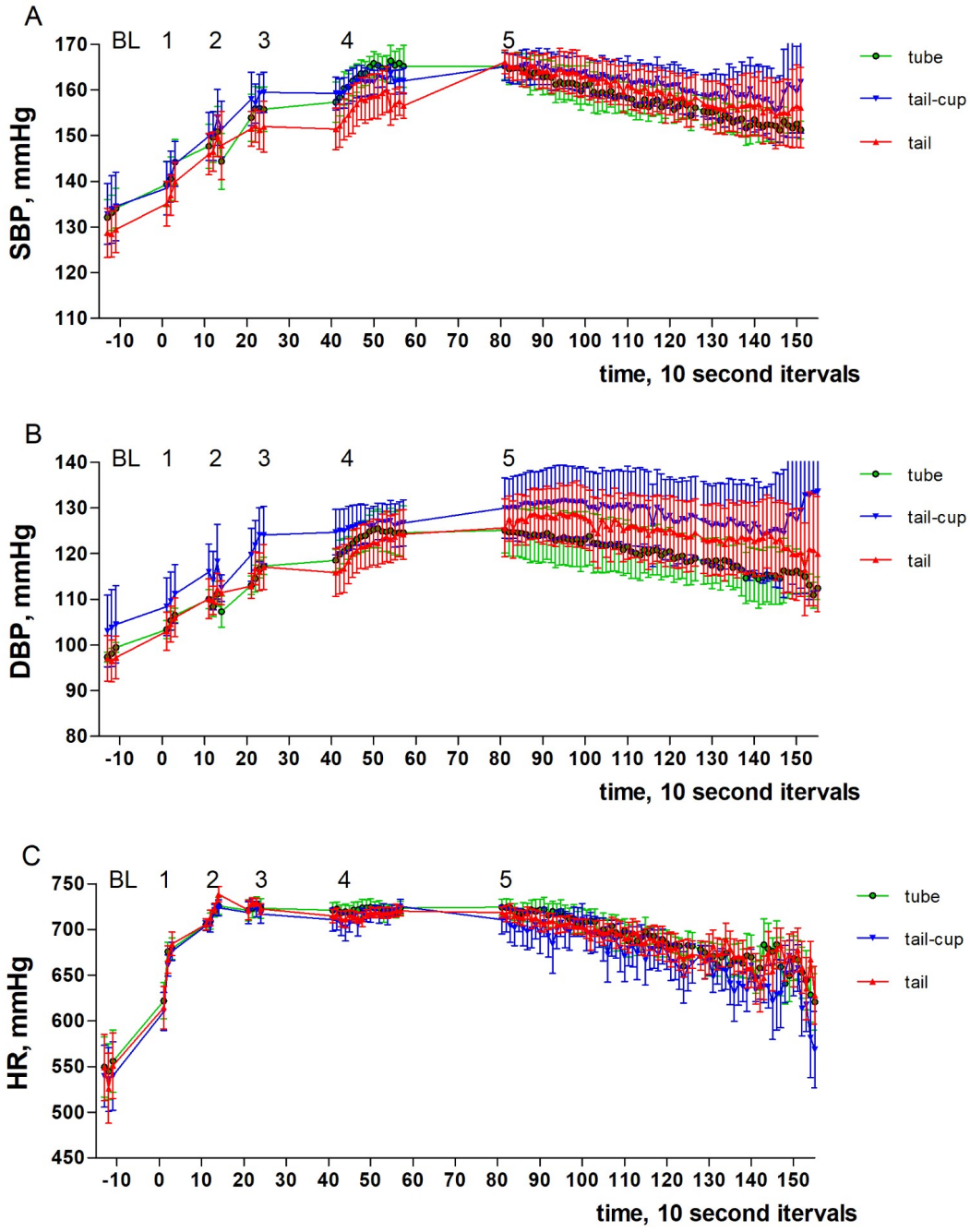


Figure 3.15. Collated data to show blood pressure changes during the handling stages of the tail-cuff protocol. *Each data point is a 10 second average (±SEM) for 6 mice over 5 recording sessions when handled by each specified technique. Time "0" is when the cage lid was lifted. BL – baseline, before the mice were disturbed, 1 – after the cage lids were removed, 2 – handling by each specified technique, 3 – placing the mouse into the tail-cuff holding tube, 4 – acclimatisation in the tube, 5 – tail-cuff recording.*

3.3.4. Habituation: the effect of repeated tail-cuff measurements on haemodynamic parameters during the recording by the tail-cuff

I did not observe significant changes in heart rate or blood pressure recordings as measured by telemetry when the tail-cuff technique was carried out for 5 consecutive days over 3 periods,

each period separated by at least 6 days rest (figure 3.16). This does not support the generally accepted hypothesis that the mice develop less stress following repeated exposure to the tail-cuff technique.

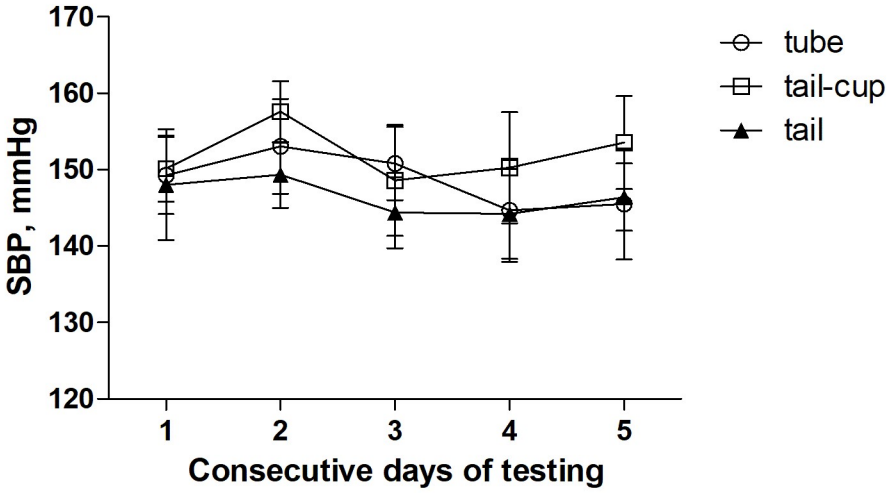


Figure 3.16. Mean systolic blood pressure (SBP) during the handling period on consecutive days of handling before the tail-cuff protocol. Each data point represents Mean ± SEM for 6 male mice (C57Bl6/J) on each of the consecutive days of handling by each technique. Fitted linear regression analysis showed that none of the fitted lines (not shown) was not significantly different from zero for any of the handling methods.

3.3.5. The effect of the scientist/handler's gender on the mouse blood pressure recordings during the tail-cuff protocol.

Blood pressure and heart rate were affected in a similar manner whether male or female researchers handled the mice for the tail-cuff throughout the tail-cuff protocol ($p>0.05$). All stages of the protocol were significantly different from baseline ($p<0.001$). Similar to the results of the previous experiment, large increases in blood pressure and heart rate compared to baseline were observed, which were maintained throughout the tail-protocol and up until 1 hour after the mice were returned to home cages (figure 3.17).

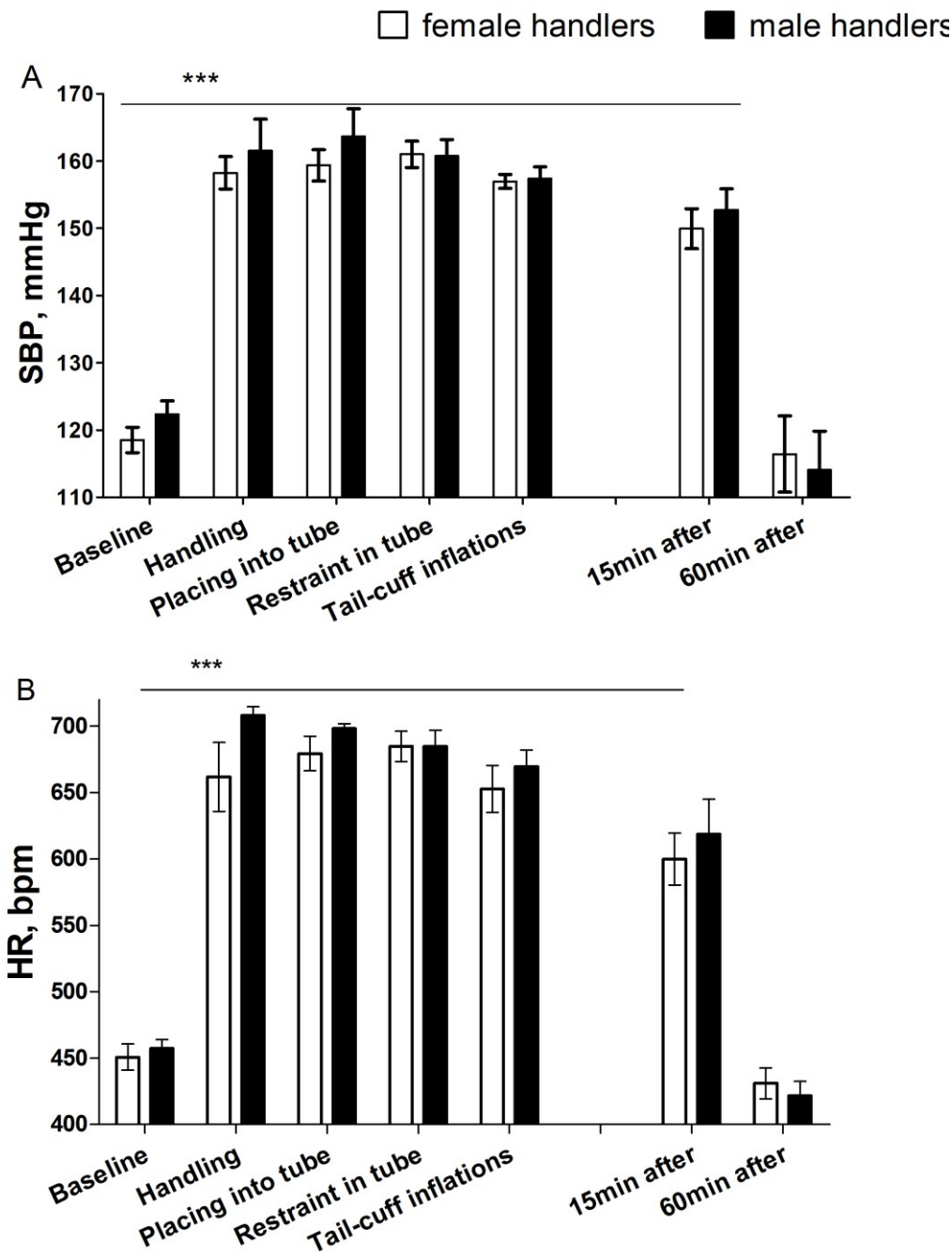


Figure 3.17. Haemodynamic changes in response to handling by male or female handlers during the stages of the tail-cuff protocol. *A, changes in systolic blood pressure (SBP) and B, heart rate (HR) during the stages of the tail-cuff protocol as measured by telemetry. Values are mean± SEM for 3 mice over 4 sessions performed by female or 3 sessions performed by male researchers on different days. Two-way ANOVA showed there is no significant difference between male (n=3) or female (n=4) handlers at any stage of the protocol (p>0.05), however each stage of the protocol is significantly different from baseline (***p<0.001), apart from the time point 60 min after the tail-cuff protocol was finished (p>0.05).*

Having conducted the experiments with other researchers, gave me the opportunity to compare the haemodynamic changes in mice in response to the presence of familiar and unfamiliar researchers, i.e. just before handling, during handling and during the rest of the tail-cuff protocol. Data in figure 3.18 shows the recordings obtained in the same mice as shown in figure 3.17 but displayed as 10-sec averages, rather than the mean values for the whole period.

Additionally, there is a period before handling took place: just after moving the cage to the platform (marked as "1" in the graph), lifting the cage lid (marked as "2"), followed by handling (marked as "3") etc.

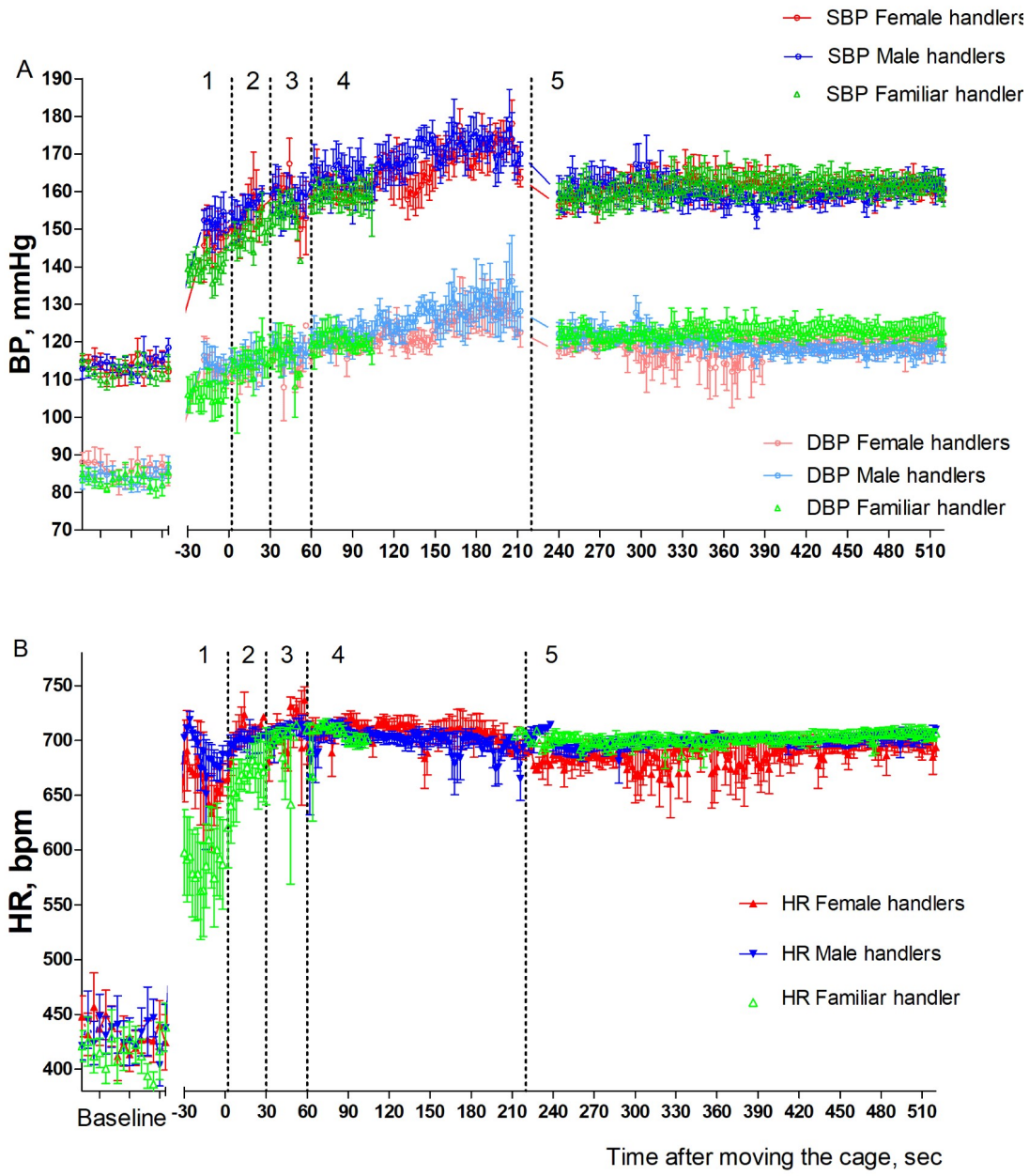


Figure 3.18. Intervention phases of the tail-cuff protocol are displayed longitudinally at the temporal resolution of 10 seconds for unfamiliar male (blue) and female (red) handlers, and familiar (female) handler (green). *A) Systolic and diastolic blood pressure and B) Heart rate. Each data point is a 10 second average reading for 3 mice over 3 to 4 days of recording when handled by familiar (male or female) or familiar (female) researchers. Baseline, 1 = moving the cage, 2 = lifting cage lid, 3 = handling, 4 = placing into the restraint/holding tube, 5 = acclimatisation in the tail-cuff holding tube.*

This data in figure 3.18 was not analysed statistically as there was missing data or differences in the lengths of the defined periods (in particular relevant to placing the mouse in to the

restraint/holding tube, “4” in figure 3.18) that were difficult to reconcile with repeated measured ANOVA. By visual comparison, there is no difference between the groups from phase “3”, i.e. from the point of handling onwards, in blood pressure or heart rate. This included the period when the measurements by the tail-cuff system were taking place and this data is not shown. Before the handling took place, there may be some minor difference in blood pressure response in phases “1” and “2” (i.e. before handling) to familiar and unfamiliar humans, but not unfamiliar male and females. There was a larger difference in heart rate during the same period between the same groups. This data suggests that mice appear to habituate to the presence of a human, however this effect appears fairly small and it disappears when handling takes place.

3.4. Discussion

The major finding in this chapter is that no significant difference was observed among the different handling techniques were found when using tail-cuff equipment alone, nor with the measurements made by telemetry. The handling by tube, tail and cup-handling described in this study are similar to those described by Hurst and West (2010). However, my approach to use telemetry recordings allowed me to establish that all the handling techniques induced similarly large increases in blood pressure and heart rate during the handling. The number of mice was not increased beyond n=6 in the light of the large impact of handling and other interventions: meaningful difference between the means would have to be appreciably large. The results of the telemetry study are considered sufficiently powered to support the observation that there is no significant difference between the handling techniques. Admittedly though a limitation is that haemodynamic parameters do not allow one to distinguish between excitement and stress.

The value of non-invasive methods to measure blood pressure in mice and rats was widely appreciated from the very beginning when smaller animals were becoming more established in cardiovascular research (Durant, 1927; Bonsmann, 1934; Griffith, 1934). Bonsmann (1934) was probably the first one to describe the technique and the apparatus for non-invasive blood pressure measurement in the unanaesthetised mice and rats. Movement of the animals was highlighted as a major challenge and, although not explicitly discussed, but implied as a marker of stress. Training, or habituating the animals to the procedure was suggested to remedy this problem. Moreover, rats were found to be more amenable to training and were considered particularly suited to the technique. Although some of the details of how the animals were restrained for the procedure are not entirely clear, it did involve placing the animals into the box.

Tail-cuff plethysmography (i.e. detection of the volume changes) was developed only a few years after for rats (Byrom and Wilson, 1938), however the measurements were made in anaesthetised

animals because the apparatus could not tolerate any movement artefacts. The technique and the apparatus were modified to include restraint, which allowed using it in conscious rats; pre-heating the animals was specified to be crucial to obtain the measurements (Williams et al., 1939). The technique was further adapted and more thoroughly characterised in mice nearly 10 years later (Wu and Visscher, 1948). Since then, tail-cuff plethysmography remains the main non-invasive method to measure blood pressure in mice and rats for nearly 80 years. Although technological progress brought several changes to the sensor technologies that are employed by the tail-cuff systems to detect and record blood pressure, the basic set-up remained largely the same: pre-heating the animal to induce sufficient vasodilation in the tail artery and restraint of the animal in the holder during the recording process, which consists of occlusion of the blood flow to the tail by means of inflating the occlusion cuff and its subsequent release that allows the return of blood flow to the tail when the blood pressure measurement takes place.

The process also involves handling, which is pre-requisite and ubiquitous in most laboratory procedures. Hurst and West (2010) showed a considerable advantage, in terms of reduced anxiety, when handling animals by tube, in a study that did not involve measuring blood pressure. By comparison, my study shows no advantage when compared with conventional tail capture in terms of acute changes in blood pressure. Additionally, in my studies, the tube handling technique did not ameliorate the impact of further handling to encourage the mice to walk into the tube, nor the restraint in the tube. Having observed the accompanying video, with the manuscript, that describes the handling techniques used by Hurst and West (2010, can be accessed via the following link: https://www.nature.com/articles/nmeth.1500) several points were realised. It was noted that the mice that were caught by the tail were not allowed to move when placed on the back of the hand. The same element was recapitulated specifically in the "tail" handling technique. Each handling technique in this study lasted approximately between 20 and 30 seconds. Mice that were caught by the tail technique in this study tended to have ears pressed against their head whilst pushing forward and rearing backwards to escape. Some mice were more difficult to capture with the tube, and once captured, also appeared anxious and looked for ways to escape the tube. On the other hand, mice often stay in the tubes and it is possible to lift them out of the cage in the tubes they are already in.

Any increase in blood pressure and heart rate that was used as a stress marker in this study, may also be precipitated by anxiety and excitement. The VPR tail-cuff system that I used throughout the project did not give a reliable estimate of heart rate, therefore this parameter, although measured, was not reported. A limitation for the blood pressure measurement by the tail-cuff technique is that it lacks temporal resolution and the blood pressure cannot be measured during

the handling process or of course before the animal is disturbed in anyway. I was also puzzled by the fact that the animals that are evidently stressed being in restraint during the tail-cuff protocol, display systolic blood pressure readings within 100 – 130 mmHg and diastolic pressure between 65 – 90 mmHg. This matter will form a question for enquiry and discussion in a later chapter. By comparison, telemetry offers high resolution recording of blood pressure and heart rate that can be taken when the mouse is in the home cage, during the period of capture and handling, placement and restraint in the tail-cuff holding tube, warming whilst in the restraint tube and thereafter during the actual recording by the tail-cuff system.

All the tested handling techniques displayed similar blood pressure and heart rate profiles at each stage, when measured by telemetry. There were significant increases in blood pressure and heart rate when the animals were handled by any of the techniques that amounted to 30 – 60 mmHg systolic blood pressure and 200-300 beats per minute for heart rate. The measured parameters tended to increase when the animals were restrained in the tube during the acclimatisation / warming period and remained elevated during the recording by the tail-cuff.

The magnitude of the changes was not entirely expected. It was decided not to pursue the investigation of the different handling techniques of the tail-cuff protocol in the light of the findings that:

1. There was no significant difference between the techniques in any of the measured parameters during handing nor afterwards

2. The handling techniques induced similarly large changes in blood pressure and heart rate

3. The effect of restraint was very large and was potentially overriding any ameliorating effect a particular handling technique could offer.

Although the impact of stressful situations or excitement on heart rate was known since ancient times, and it was highlighted some 80 years ago that the animals struggled during the blood pressure measurement protocol, the issue of stress and the impact this can have on blood pressure was not discussed in the earlier studies (Bonsmann, 1934; Williams et al., 1939; Shuler et al., 1944; Wu and Visscher, 1948), nor in the studies that followed (Henry et al., 1965). It was however recommended to habituate the animals to the procedure. The references to the fact that the animals experience stress beyond the notion of “restlessness” during the tail-cuff protocol appeared in the literature in the 1960s (Henry et al., 1967; Buñag, 1983), however training the animals to the procedure is still deemed sufficient to obtain reliable measurements

(Feng et al., 2008, 2009; Daugherty et al., 2009). On the other hand, my results indicate that habituation does not offer a tangible improvement.

Handling is made up of many variables including the specific technique of how the mouse is captured and a range of other factors involved with the handler and the environment, such as olfactory signals (Sorge et al., 2014) and noise (Reynolds et al., 2010). Sound and smell signals are perceived differently by mice and humans and thus potentially present a source of variability that may be difficult to control. An interesting finding by Sorge et al (Sorge et al., 2014) revealed that handler's gender can be an important variable in behavioural tests in that male researchers are more stressful to male and even more so to female mice. This study showed that mice (and rats) actively suppress acute pain signals when exposed to male hormones olfactory stimuli: similar effect was observed when exposed to human and other mammal androgen secretions.

These observed stress responses were accompanied with significant increases in stress hormones and other stress biomarkers in the mice.

A study Gouveia and Hurst (2013) further stress the importance of olfactory cues in mice. This study shows that familiar smells can be used to ameliorate the anxiety associated with laboratory procedures in mice: handling mice using a tunnel that was previously kept in the same cage with the mouse can further reduce handling-induced anxiety level. In spite of the complexity of the handling phenomena, the range of studies by the group (Hurst and West, 2010; Gouveia and Hurst, 2013, 2017) highlighted that the manner that the mouse is captured is crucial and has far-reaching consequences on how the mouse reacts to other stressful interventions such as restraint and has other profound effects on the mouse's behaviour. Although individual and strain variability can be important how mice can respond to handling, the positive effect of non-aversive handling has substantial positive effect on all tested strains of mice of both genders.

Tail-cuff protocol involves restraint, which is undoubtedly stressful to mice and can override any ameliorative impact of handling. Sorge et al (2014) showed that in fact 15 minutes of restraint induces similar or even larger increase in stress biomarkers as the exposure to male odour. However, in our hands, male scientists induced similar increases in blood pressure and heart rate in the mice, as that induced by female scientists during the handling and during the ensuing stages of the tail-cuff protocol.

To conclude, these studies highlight the complex nature of the handling process and how this can be perceived by the mouse. Undoubtedly the handling technique, experimenter's gender, the environment, habituation, mouse's strain and gender have been shown to affect the stress and

anxiety level in the mouse and it its turn affects the mouse's behavioural and physiological responses. However when the selected handling techniques were tested in the context of the tail-cuff protocol, neither of the techniques offered a significant advantage to reduce the observed increase in the blood pressure and heart rate during the handling or whilst in restraint.

Chapter 4.

The Effect of the Different Stages of the Tail-Cuff Protocol on the Blood Pressure and Core Temperature in the Mouse

4.1. Introduction

The tail-cuff procedure is a valuable measurement system used routinely in cardiovascular research, especially for mice as previously discussed. However, it involves a range of interventions that have been shown to be stressful to laboratory animals, including mice (Balcombe et al., 2004). The tail-cuff protocol to measure blood pressure in the mouse typically includes the following steps. Firstly, the mice are transferred (in their home cages) to a designated area where blood pressure measurement takes place. This should be a quiet warm room that could be otherwise suitable for behavioural testing. Secondly, following an acclimatisation period in the procedure room, the mice are lifted from their home cages and are placed in the tail-cuff holding tubes, which are kept on the heating platform during the experiment. The purpose of the holding tube is to restrain the mouse for the duration of the blood pressure measurement protocol. It is very important that the mouse is warmed to around 32-35°C to ensure sufficient blood flow to the tail, from which the blood pressure measurements are taken, hence the use of the heating platform. Finally, the actual blood pressure measurement consists of a series of inflation and deflation cycles of the cuffs that are placed on the mouse's tail (Daugherty et al., 2009).

It is easy to appreciate that the whole range of interventions associated with the tail-cuff protocol may have a direct impact on haemodynamics: starting from the essential human presence in the room, required for handling the mouse, and initiating as well as supervising the restraint process (Gross and Luft, 2003; Balcombe et al., 2004; Kramer et al., 2004; Reynolds et al., 2010; Batchu et al., 2015), also operating the machinery, so that warming and cuff inflations in the context of the tail-cuff protocol can occur (Buñag and Butterfield, 1982; Buñag, 1983; Irvine et al., 1997; Whitesall et al., 2004). Although each of these components and the human presence are understood as important for the procedure; as well as possibly, being limitations of the tail-cuff protocol, no formal investigation of the actual impact of the different components on the cardiovascular system of the mouse has been undertaken to my knowledge.

The tail-cuff technique is valuable in that it is an easier and less expensive alternative to telemetry and essential when it is not possible to use telemetry for ethical or technical reasons.

There is, therefore, an urgent need to understand what effect each of the different components has on the measured parameters and if there is a way to ameliorate these unwanted effects.

The aim of this chapter was to:

- Investigate the effect of each step of the tail cuff technique on blood pressure, heart rate, activity and core body temperature, as measured by telemetry.
- Examine the effect of turning on the telemetry equipment on cardiovascular parameters, activity and core body temperature
- Examine if mice habituate to specific disturbances such as turning on the telemetry equipment, handling, restraint and the tail-cuff technique (that includes all the aforementioned disturbances)
- Determine the variation of blood pressure, heart rate, core body temperature and activity in free moving mice in their home cages in the absence of human presence (effect of diurnal variation and activity not otherwise influenced by human intervention).

4.2. Protocol details including experimental design and development

4.2.1. Animals

A total of 8 male (n=8*)* and 15 female (n=15) C57Bl6/J mice were used in this phase of the project. Additionally data for a further 6 male C57Bl6/J telemetry-implanted mice that were used in the experiments described in the previous chapter is also included in the analysis shown in this chapter. The number and gender of mice used in each experiment are specified in each case. All C57Bl6/J mice used in these experiments were bred in house. The age range of the mice used in these experiments at the start of the procedures was between 12 and 15 weeks.

4.2.2. Measuring blood pressure and core body temperature

Blood pressure, heart rate and activity were measured using radiotelemetry [PA-C10; Data Science International (DSI), St. Paul, MN, USA] as described in Chapter 2, section 1.4.2. The protocol for tail-cuff plethysmography to measure blood pressure was as described in Chapter 2, section 1.4.1. Core body temperature was measured using radiotelemetry transmitter (TA10TA-F10; DSI, St. Paul, MN, USA] as described in Chapter 2, section 1.2.2. All devices were surgically implanted as described in the relevant sections in chapter 2.2.

4.2.3. An in-depth study of the components of the tail-cuff protocol on blood pressure and core body temperature

Male (n=2) and female (n=2) C57Bl/6 mice implanted with blood pressure telemetry probes were used in the experiments to measure the effect of the different components of the tail-cuff protocol on blood pressure. Female C57Bl/6 mice (n=4) implanted with temperature telemetry probes were used in the experiments to measure the effects of the different components of the tail-cuff protocol on core body temperature.

The telemetry data (temperature or cardiovascular) was collected continuously at 10 sec segments and presented as 1-minute averages, or other periods as specified on individual graphs.

The following components of the tail-cuff protocol were the focus of investigation in this series of experiments: A) presence of the researcher in the room, B) moving the mouse in the cage next to the equipment, C) handling, D) restraint without warming, E) restraint with warming to 32-35°C and finally F) restraint with warming to 32-35°C and measuring blood pressure by the tail-cuff. Telemetry was used to learn of the effect of these interventions on mouse central blood pressure, heart rate, core temperature and activity where applicable.

The following experimental procedures were performed on separate days, repeated on at least two occasions for each animal. These mice had been handled and habituated to the tail-cuff protocol on at least 4 occasions before being implanted with telemetry probes. The mice were also handled after the surgery to monitor for recovery.

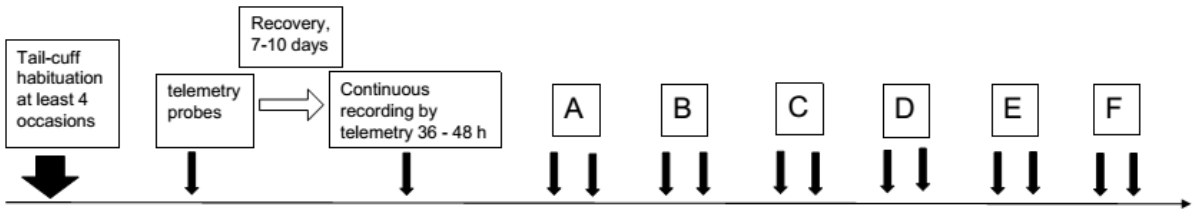


Diagram 4.1 Outline of the experimental procedures to measure the effect of the defined stages of the tail-cuff protocol (A-F) on haemodynamics or core body temperature of the mouse; n=2 male and n=2 female C57Bl6/J mice.

A. The researcher entered and remained in the room for approximately 3 minutes.

B. The mouse in the home cage was moved close to the platform next to the tail-cuff machine and left for 15 min without handling.

C. To test the effect of handling alone, the mice were picked up from the home cage using the tail-cup technique, held in the hand for approximately 30 sec and then returned to the home cage for further recording over 15 min.

D. For the effect of restraint without heating, the mice were restrained in the tube for 15 minutes at ambient temperature (22-24°C) without pre-heating the tube or the underlying platform.

E. To test the effect of restraint and heating, the mice were restrained for 15 minutes in the tubes pre-heated and maintained at 33-35°C.

F. The final step involved testing the effect of the complete tail-cuff procedure that typically lasted 15 minutes. For this the mice were removed from the home cages, placed in the warmed restraint tube on the pre-heated platform and subjected to tail-cuff recording after 5 min of acclimatisation on the platform. Each recording session consisted of 15 inflation and deflation cycles of the cuff over the mouse's tail, with the first 5 cycles being used as "acclimatisation" cycles and not used in the analysis.

In the case of the "restraint and heating" and "tail-cuff procedure" experiments, the temperature of the mouse's tail and the mouse's body were monitored using an infra-red thermometer and maintained within the specified range using an infra-red lamp.

"Baseline" is a term that is commonly used to describe the level and expected variation in a measured parameter before the changes in the controlled variable. Baseline measurements were made on each day of the experiment. In this series of experiments, the 15-minute periods when the mice were at rest and when no known external disturbances were evident were chosen as the reference baseline periods. This typically corresponded to a period approximately 30 minutes after the telemetry probes were switched on each day of experiment.

4.2.4. The effect of other disturbances

Turning on the telemetry probes appears to affect blood pressure and activity in mice. I chose to analyse the occasions when telemetry was switched on for the first time after a period when the interventions were limited to welfare checks and where at least one hour of recording free of any disturbances was available after the probes were switched on. I had suitable data available from other experiments for both male (n=5) and female (n=6) C57Bl/6 mice that were implanted with cardiovascular telemetry and four female mice that were implanted with the temperature telemetry.

4.2.5. Habituation to turning on the telemetry probes, handling, restraint and the tail-cuff recording cycles.

Data from the series of experiments that compared the effect of the different handling techniques offered the suitable longitudinal data to understand if there is evidence that mice respond to repeated tail-cuff protocol with less elevation in blood pressure and heart rate. The data was arranged in chronological order, disregarding the type of handling that the mice were subjected to on that week of testing. The same data set was also analysed if mice respond with a smaller increase in blood pressure and heart rate to handling before restraint took place.

Turning on the telemetry probes was scrutinised for the element of habituation. It was noted that maximal increase in any parameters is achieved within 15 minutes of switching on the probes, therefore this period was chosen to calculate the average and compare how these values change over successive recording sessions.

4.2.6. Normal variation of haemodynamics and core body temperature associated with circadian rhythms and activity in undisturbed free-moving male and female C57Bl6/J mice in their home cages

Normal level and variation of physiological parameters are commonly referred to as “baseline”. To understand the normal level and variation of cardiovascular parameters and core body temperature in mice, these parameters were recorded for at least 24 hours following recovery from the surgery (10 days) in all mice that were implanted with the telemetry devices, taking care to minimise any disturbance to the animals. In total, data from 10 male and 11 female C57Bl/6 mice was available and presented in this section. Only 4 female C57Bl/6 mice were implanted with temperature telemetry.

Cardiovascular parameters, core temperature and activity were recorded for 10 seconds at 10minute intervals and the average values for these periods were recorded by the software. These values were used as estimates for average core body temperature and activity for each respective 10-minute period and were further used to calculate hourly and diurnal (light and dark) average values for the measured parameters. In some animals, cardiovascular parameters and activity were measured continuously and the software calculated 2-second or 10-second averages for each of the parameters that were used to calculate hourly and “light” and “dark” cycle averages. Baseline recordings over at least 36 hours were obtained for all animals.

4.2.7. Statistical analysis and considerations

Based on the results of the telemetry-based handling study (described in chapter 3, sections 3.3), I anticipate large difference of the interventions compared to baseline. Since

the aim is to investigate the effect of these different interventions, I am powering the study to see the effect of each intervention compared to baseline and not the potential difference between the interventions. Therefore I anticipate large effect size and a small number of animals (such as n=4) may be sufficient to show the statistical difference.

Statistical analysis and figures were realised using GraphPad Prism 5.0. Further details of the analysis and data presentation are described for each figure in the results section.

4.3. Results

4.3.1. An in-depth temporal analysis of the effect of person entering room, moving cages, handling and tail-cuff techniques on telemetry measurements of blood pressure, heart rate, activity and temperature.

The purpose of this series of experiments was to analyse the different components of the tail-cuff protocol that may be stressful to the mouse, starting with the presence of the investigator in the room, moving the cage, handling the mouse, restraint in the tube with and without the heating and finally restraint with heating and the cyclic compressions with the tail-cuff.

The average blood pressure (SBP±SD/DBP±SD) for 4 mice during the undisturbed period before the interventions took place was 113.5±10.0/85.3±10.4 mmHg, the heart rate was 495.3±19.4 beats per minute and activity measured 1.2±1.1 counts for all the days when the interventions took place. The presence of the investigator in the room did not cause significant perturbations in blood pressure (figure 4.1): SBP was 121±14/92±14 mmHg at the start of the period when researcher was present in the room and 126±11/92±7 (SBP/DBP±SD) by the time the researcher left. The heart rate and activity were 533.9±19.9 - 557.8±13.0 bpm and 3.7±0.8 – 0.8±0.8 counts at the same respective periods. This was not significantly different from the preceding period that was used as “baseline”. Therefore, this intervention was equated to “background noise” and was used to compare other interventions to.

Moving the mouse in its home cage had immediate, although transient, impact on blood pressure and heart rate (figure 4.1). Systolic and diastolic pressure rose to 140±16/104±10 mmHg and heart rate to 682±23 bpm. Activity rose. The increase in heart rate and systolic blood pressure became significant from the first and second (###$p<0.0001$, ##$p<0.001$) minutes respectively. Activity level also rose significantly at the second minute after the cage was moved (##$p<0.001$, figure 4.1D), reaching 5 and 23 counts in the first and second minutes respectively and remained slightly elevated throughout. The increase in diastolic blood pressure was less clearly defined by

the intervention and became significant mostly by the virtue of the relative decrease in diastolic blood pressure during the respective comparator period before the intervention took place.

Handling induced clearly defined changes in all the measured parameters. The increases in systolic blood pressure, heart rate and activity (***p<0.0001 figure 4.1A and C, **p<0.001 figure 4.1 D) became significant from the first minute, whereas the increase in diastolic blood pressure became significant from the second minute (**p<0.001 figure 4.1B). Blood pressure reached

149±11/111±6 mmHg (SBP±SD/DBP±SD) during the first minute and peaked at 152±10/112±7 mmHg at the second minute, whereas heart rate reached 730±5 bpm during the first minute, reducing to 575±27 bpm during the 15th minute.

Although handling induced more distinctive changes in the measured parameters, “handling” and “moving cage” interventions were not significantly different from each other; following a brief peak immediately after either of the interventions was over, all the measured parameters started to decrease and returned to the level that was not significantly different from baseline within the 15-minute period.

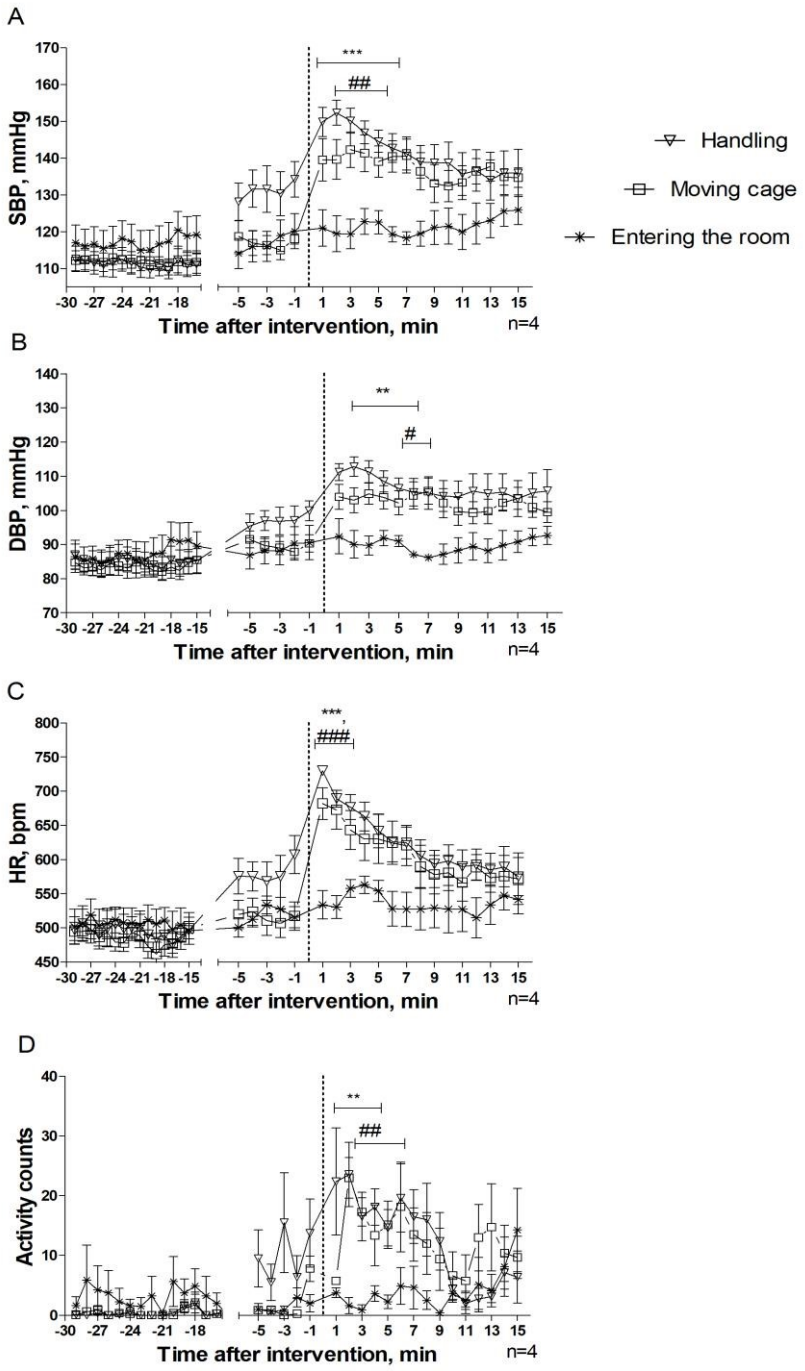


Figure 4.1. Comparing/Measuring the effect of entering the room, moving the cage and handling interventions on A) Systolic blood pressure (SBP), B) Diastolic blood pressure (DBP), C) heart rate (HR) and D) activity of the mouse. *Each data point represents an average value (±SEM) for 4 mice over one-minute periods before and 15 minutes following an intervention. The 1st minute on the x-axis typically represents the 1st minute after the handling and the cage movement were completed. Two-Way RM ANOVA test-followed by Bonferroni post-test significant results are shown as follows: handling (*p<0.05) and cage movement (#<0.05) induced significant increase compared to baseline in the measured parameters for certain periods following interventions as shown in the graphs.*

The average core temperatures and activity during the baseline period before interventions were 36.4±0.5°C and 1.0±2.5 counts (figure 4.2). Although this period was chosen to represent a period when animals were at rest, there was significant difference at the start of the period that

preceded the “handling” (&p<0.05) at -30th and -29th minutes compared to the baseline period preceding the “moving cage” intervention. “Entering the room” intervention, on the other hand, produced no appreciable effects on core body temperature or activity. The core temperature was 36.2±0.5°C with no activity during the first minute, thereafter reaching 36.3±0.6°C and 3.0±6.2 activity counts during the 3rd minute and remained at the similar level throughout the recording period for this intervention. “Entering the room” intervention was used as a no-effect intervention for comparison in statistical analysis in preference to the baseline periods to simplify the procedure.

Cage movement and handling caused large and clearly defined changes in core temperature, as well as activity. Following cage movement, core temperature started to rise steadily, from 36.2±0.5°C at the second minute, approaching 37.0±0.4°C by the 6th minute and stayed at this level at least until the end of the recording session. The rise in core body temperature became significantly different compared to baseline and the intervention that showed no effect (“entering the room”) from the 6th minute (#p<0.05, ##p<0.01) until the end of the session. Cage movement caused a spike in activity that averaged 19.4±9.3 counts during the first minute that remained significantly different for 3 minutes compared to the no effect level (###p>0.0001), that later diminished and started to approach zero (0.2±0.4 counts) towards the end of the experiment.

Handling caused a large increase in core temperature from the 1st minute, 37.1±0.3°C (*p<0.05 compared to the corresponding 1st minute after the researcher entered the room). The core temperature continued to rise and peaked at 38.0±0.3°C at the 6th minute and it remained significantly different from the “entering the room” intervention throughout (***p<0.001 minutes 2 – 15). Furthermore, core temperature following handling remained significantly different from the core temperature following cage movement between the 1st and the 9th minutes (&&p<0.01 on average). Activity was overall similarly higher following handling and moving the cage with this effect being significant between the 2nd and 11th minutes compared to “entering the room” intervention (**, ##p<0.01).

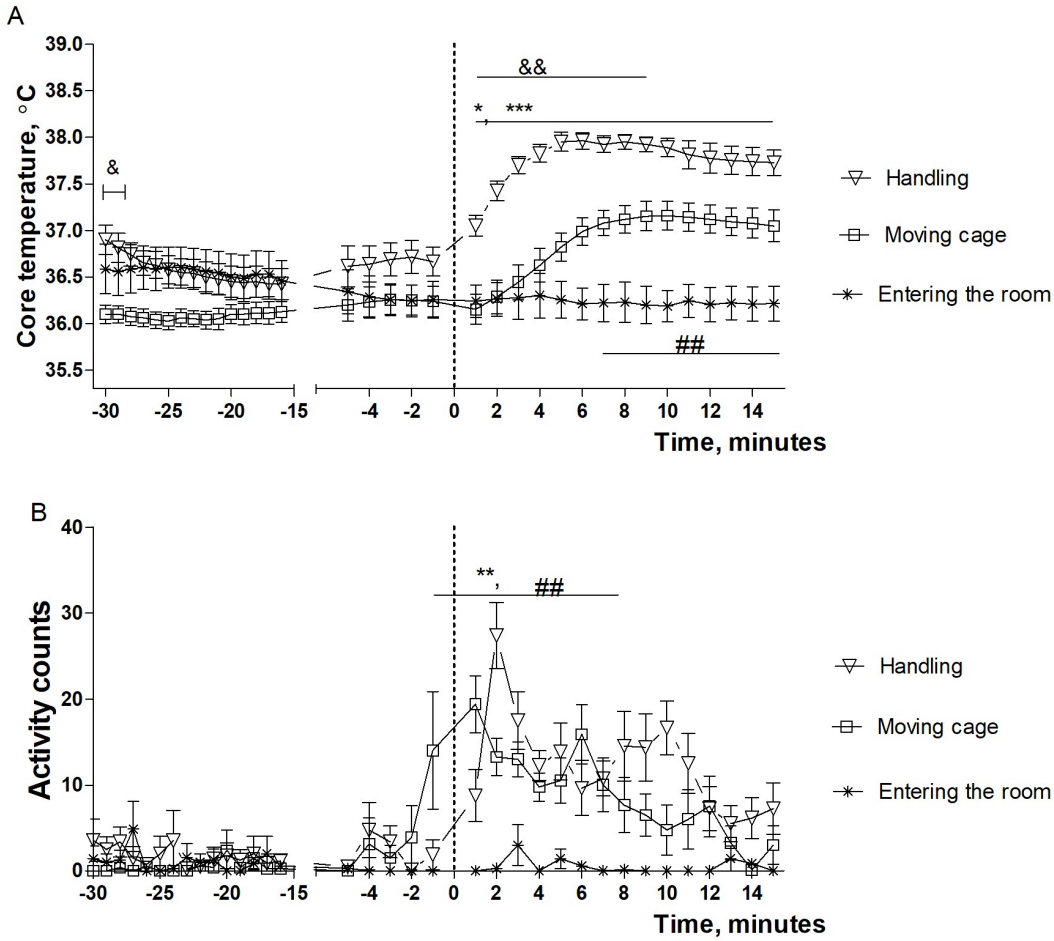


Figure 4.2. Comparing the effect of entering the room, moving the cage and handling interventions on A) core body temperature and B) activity of the mouse. *Each data point represents an average value (±SEM) for 4 mice over one-minute periods before and up to 15 minutes following an intervention. The 1st minute on the x-axis typically represents the 1st minute after the handling and the cage movement were completed. Significant results of the two-Way RM ANOVA test and Bonferroni post-tests are shown as follows: A) handling (*p<0.05 after the 1st minute, thereafter ***p<0.001) between the 1 - 15 minutes, and moving the cage (##p<0.001) after the 7th minute compared to "entering the room" intervention; (&&p<0.01) for handling between the 1st and 9th minutes as compared to moving the cage intervention. B)Activity was significantly higher following both handling and cage movement from the "entering the room" intervention between 1st and 11th minutes (**,##p<0.01).*

4.3.2. The effect of restraint and heating on blood pressure, heart rate and core body temperature

All interventions that involved restraint induced highly pronounced and sustained elevations in blood pressure and heart rate (figure 4.3). All these changes were significantly different compared to the period before the interventions were initiated (later referred to as "baseline" period in the context of this series of experiments), however all interventions were similar to each other in their effect on the measured haemodynamic parameters. The only significant difference between the different treatments was at -5th minute between the "No heat" and "Tail-cuff" interventions.

In the case of restraint without heating, SBP range was 159.7±11.4 mmHg (Mean ± SD) at the first minute to 165±11.7 mmHg at the 8th and 9th minutes. At the last minute, SBP was 161.6±9.4 mmHg. Diastolic pressure remained level, 119.6±7.3mmHg mmHg at the start and 19.6±8.3mmHg at the end of the procedure, marginally peaking at 122.0±8.6 mmHg on the 13th minute. The average heart rate was 722.0±16.8 beats per minute (bpm) at start and 722.1±28.4 bpm at the end of the restraint period.

When the restraint tubes were pre-heated before the mice were placed into the tubes, SBP and DBP were 161.5±12.5 and 121.8±10.7mmHg respectively during the first minute, which was also the maximum value, thereafter reducing to 156.7±7.6 and 116.7 mmHg at the end of the 15minute period. The heart rate was 707.2±9.3 bpm at the start and 690.6±23.2 bpm at the end of the procedure.

In the experiments when tail-cuff recording cycles were initiated on the 5th minute of restraint, no augmentation of the blood pressure that could be attributed to the tail-cuff inflation cycles was observed. Average systolic and diastolic blood pressure were 160.8±7.8 and 121.4 mmHg respectively during the first minute, 158.6±11.6 and 121.5±12.7 mmHg during the 5th minute, and 156.3±10.2 and 116.6±10.0 mmHg during the 15th minute of the recording session when tail-cuff cycles took place. The heart rate was 716.2±50.8 during the first minute, 692.7±47.1 during the 5th minute when the tail-cuff cycles were initiated, and 702.3±23.9 during the last, i.e. 15th minute of the restraint.

The time count started as soon as mice were placed into the restraint, i.e. 1st minute on the X-axis (figure 4.3) represents the first minute the mice spent in the restraint. Mice were caught and placed into the restraint between “-2 ...-1” minutes, therefore the -5…-1 minutes as shown in figure 4.3 recapitulate the compound effect of cage movement and handling that are shown in the figure 4.1 (and 4.2 for the effect on core body temperature). Blood pressure of 150.9±13.3/114.2±8.2 and heart rate 687.1±62.3 very closely reproduce the results obtained following handling.

Two-Way RM ANOVA revealed no significant differences between restraint periods “No heat”, “+ heat”, and “Tail-cuff” for blood pressure or heart rate, however there was significant difference 5 minutes before the restraint period between the “No heat”, “+heat” and “Tail-cuff” treatment group (*p<0.05, **p<0.01, ***p<0.001 as indicated in the figure 4.3A-C). All of the measured parameters from -3rd minute (before the restraint) and throughout the restraint period were significantly different from those measured during the baseline period (###p<0.001).

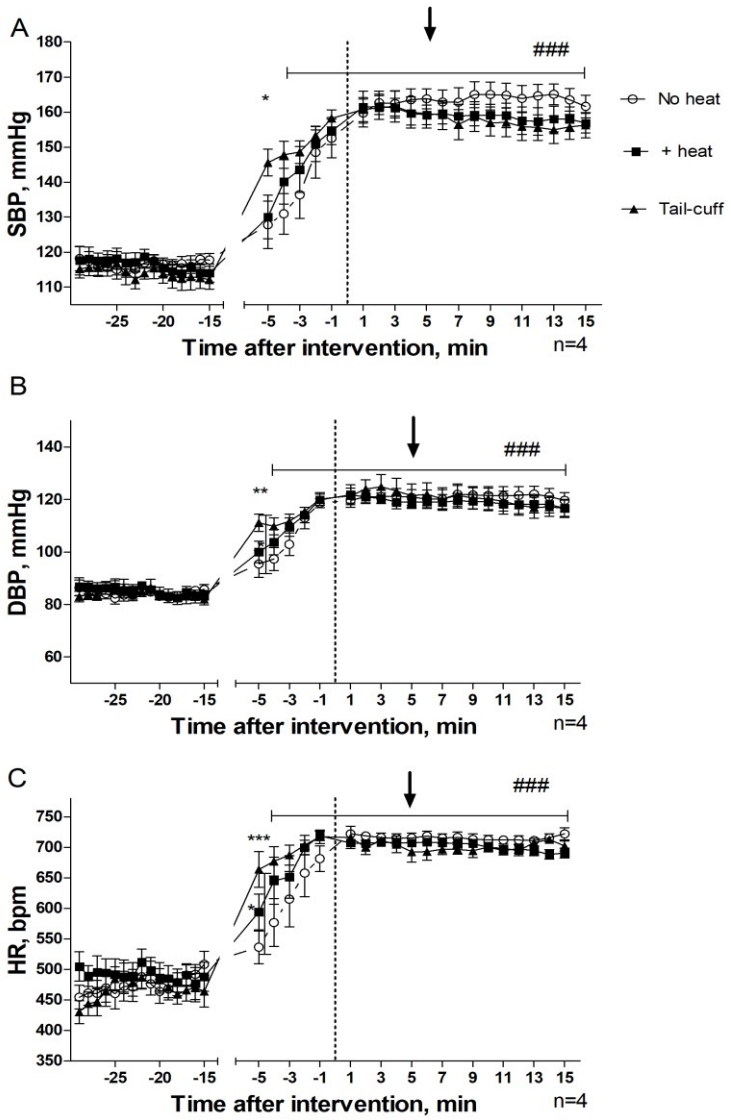


Figure 4.3. Measuring and comparing the effect of restraint with and without heating, with and without the tail-cuff inflations on A) Systolic blood pressure (SBP), B) Diastolic blood pressure (DBP) and C) heart rate (HR). *The arrow indicates the start of the recording cycles that typically were initiated on the 5th minute following the placement of the mouse into the tube. Each data point represents Mean ± SD for 4 C57Bl/6 mice (males n=2 and females n=2) over 2-3 sessions for each mouse. Two-Way RM ANOVA and Bonferroni post-test significant results are indicated for all treatment groups ("No heat", "+ heat", and "Tail-cuff")*p<0.05, **p<0.01, ***p<0.001 as indicated in the figures) and are significant from -3rd minute (before the restraint) and throughout the restraint compared to the baseline period.*

The comparison of the effect of the interventions that involved restraint on core temperature was made to the period before the interventions on each day of the experiment (referred to as "baseline" in this series of experiments) and to each other. Likewise, the interventions that involved restraint in pre-heated tubes induced progressive and significant increase in the core body temperature (figure 4.4).

On the occasions when the tubes and the platform were pre-heated to 33-36°C, the core body temperature rose steadily from 36.9±0.3°C (pre-heating only, no tail-cuff) and 37.1±0.2°C

(preheating and tail-cuff) during the first minute, to 37.5±0.2°C and 37.7±0.2°C during the 4th minute for each intervention type respectively. After this point the temperature increase continued, albeit at a slower rate, and reached the maximum at 37.9±0.3°C on the 14th minute when no tail-cuff cycles took place and 38.2±0.2°C on the 12th minute when the tail-cuff cycles took place. There was no significant difference between these two interventions at any time, neither before, nor after the tail-cuff occlusion cycles were initiated at the 5th minute. Both intervention types are significantly different from baseline (* and #p<0.05 during the first minute and *** and ###p<0.0001 for the pre-heating only and tail-cuff interventions respectively).

On the occasions when the holding tubes and the platform were kept at ambient temperature, i.e. in the range of 22.5-25.0°C before mice were placed into the restraint and no heating was used thereafter, the core temperature was 37.0±0.4°C on average during the first minute of restraint, which was remarkably similar to the interventions when pre-heating was used. However, when mice were restrained in the tubes without heating, the core body temperature tended to decrease and reached 36.5±0.2°C during the 4th minute. It started to increase thereafter for the rest of the recording period, reaching on average 37.2±0.2 during the 15th minute and still tended to rise. The core body temperature during this type of intervention was only significantly different from baseline during the 1st minute (&p<0.05) and after the 9th minute (&p<0.05 on the 9th minute and &&p<0.001 thereafter; figure 4.4).

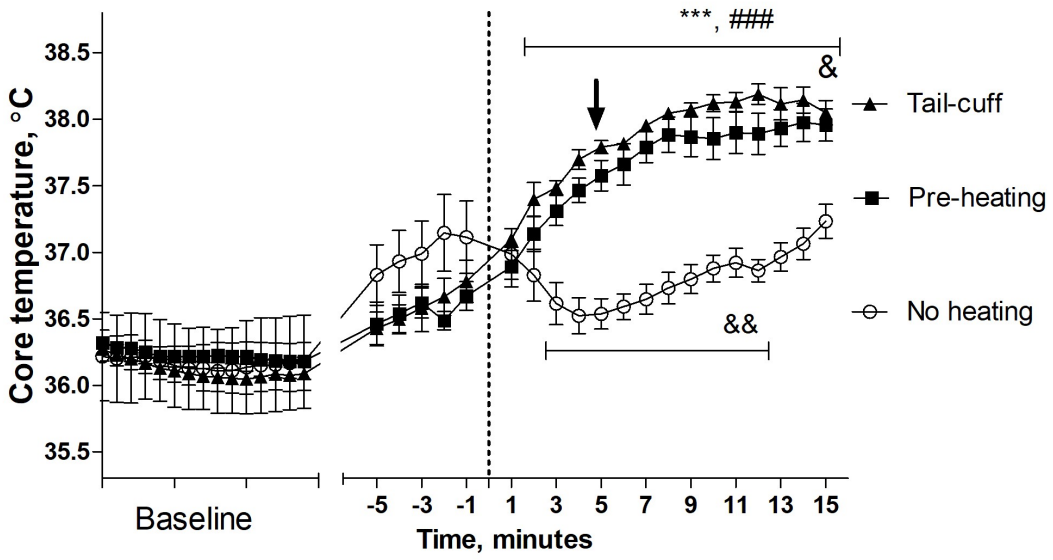


Figure 4.4. Comparing/Measuring the effect of restraint with and without heating and tail-cuff inflations on Core body temperature of the mouse. *The arrow indicates the start of the recording cycles that were initiated on the 5th minute following the placement of the mouse into the tube. Each data point represents Mean ± SEM for 4 C57Bl/6 female mice over 2-3 sessions for each mouse. Tail-cuff and restraint with pre-heating were significantly different from the baseline period (***, ###p<0.001 respectively) from the second minute in restraint. The restraint with No heating was significantly different from the baseline period during the 15th minute (&p<0.05) and it was significantly different from the other two interventions between the 3rd and 11th minutes (&&p<0.01).*

All interventions, apart from the researcher entering and staying in the room for 1-3 minutes, induced clearly defined changes in blood pressure, heart rate, activity and core body temperature in mice. Interventions that involved restraint induced the largest and sustained increases in the measured parameters. Changes in core body temperature (figure 4.2) developed more slowly than the changes in the cardiovascular parameters (figure 4.1). Blood pressure and heart rate increases induced by moving the cage and handling tended to return to baseline by the 15th minute the intervention was over. Rises in the core body temperature induced by the same interventions took longer to develop, however it has not been formally studied how long the measured parameters take to return to the baseline level. I shall examine in the following section the amount of time it takes for the haemodynamic parameters and core body temperature to return to baseline following the tail-cuff technique.

4.3.3. A temporal study of the recovery of cardiovascular parameters and core body temperature, as measured by telemetry during and after the tail cuff technique.

The previous experiments focussed on the haemodynamic and core body temperature effects during the time the interventions took place and/or within the 15-minute period that the tail-cuff protocol typically lasted for a mouse. Analysis of the recorded parameters following the

completion of the tail-cuff protocol was not the focus of the experiments which investigated the effect of the different handling techniques or the components of the tail-cuff protocol on haemodynamics and core body temperature. The recordings after the tail-cuff protocol was complete were available following almost all sessions, albeit for different lengths of time. The telemetry recordings obtained for the tail-cuff protocol in these experiments indicate that blood pressure and core body temperature typically returned to the pre-intervention level within 2 hours. Cardiovascular recordings to cover 2 hours following the tail-cuff protocol were available for 3 male mice on 5 successive occasions and temperature data was typically available for the 4 female mice on 3 successive occasions.

Recording sessions using blood pressure and temperature telemetry that also include the 2-hour recovery period following the tail-cuff are represented in figures 4.5 and 4.6 respectively. Figure 4.5 shows the same data as figure 3.17 and 3.18 in the previous chapter. That data was obtained as part of the study that investigated the impact of the handler's gender on the mouse's haemodynamic changes during the tail-cuff protocol. Although there may have been some difference in the way the male and female researchers impact the haemodynamic changes in mice, these changes were shown to be similar after handling took place. It was apparent then (figure 3.17 and 3.18) that the handler's gender, or whether the handler was familiar to mice, had no impact on how the mice reacted to the restraint. Therefore, I do not believe that the handler's gender affected the way the mice recovered after the tail-cuff protocol. In the absence of other systematic data (where several repeats were available for the same mouse under similar conditions) that included the period of at least one hour recovery, I decided to include the data obtained as part of the gender study in this chapter as well to discuss the recovery of the recorded parameters.

The figures show the 15-minute "baseline" period on each day before the tail-cuff recordings took place, which is marked as "Baseline". The 10-minute period shown before the -15 minute on the X-axis included the time immediately before lifting the cage lid and handling the mouse to place the mouse into the pre-heated restraint tube. The mice were typically in the restraint tubes at the time-point represented as "-15" minute on the X-axes for both cardiovascular and core temperature recordings. The tail-cuff cycles, i.e. the cycles of compression and release of the occlusion cuff over the mouse's tail, were initiated at approximately the "-10" minute on the x-axes (indicated with arrow). Mice started being released from the restraint tubes at around "0" time-point, with the cages put back onto the holding racks shortly after and the mice allowed to recover.

The blood pressure rose rapidly and peaked when the animals were being handled and placed into the restraint tubes, 167.4±8.4/122.4±8.8 mmHg at -19th minute (SBP±SD/DBP±SD). During the first minute in restraint the blood pressure was 161.4±5.1/119.6±6.9 mmHg and 153.1±3.5/116.0±7.5 mmHg after the last minute in restraint. The systolic blood pressure reduced by 8 mmHg towards the end of the restraint period, while the diastolic blood pressure changes were masked by the high variability.

The heart rate peaked at -16th minute at 701.5±8.8 bpm; thereafter it tended to decrease, however it became more variable among the mice, such that it was 693.3±18.5 bpm after the first minute in restraint and 643.7±50.9 bpm the minute before the mice were released from restraint.

Once the mice were released from the restraint after the tail-cuff protocol was complete (figure 4.5), the heart rate did not immediately decrease, but somewhat peaked at around 650±40 bpm mark for approximately 5 minutes and thereafter continues to decrease in a stepwise manner: plateau periods of 3-8 minutes were followed by a relatively sharp steady decreases until the lowest average value of 402±50 bpm was reached during the 48th minute after the mice were returned to their home cages. The heart rate reached the "baseline" level after the 45th minute and it largely remained around the similar level to baseline, albeit with larger variation. Although the blood pressure reached the resting level before 45 minutes following the completion of the protocol, the blood pressure remained highly variable until one hour passed.

The sharp rise in activity before the tail-cuff protocol (before -15 minute) largely due to mice escaping capture, followed by movement when mice were carried and placed into restraint. Some of this movement was not due to the mouse movement, but rather the mouse being carried, therefore these activity counts would be artefacts. It was difficult to separate the true readings from the artefacts. The pattern seen is regarded as representative of the visual observation of the mouse's behaviour before and during the placement into the tubes, therefore the data shown Is believed to be a fair estimation of the mouse's activity at this time.

Once in the restraint, the mouse's movement was restricted as intended by the purpose of the restraint and typically remained as "0" throughout the period of restraint. In some cases, however, the mice remained restless while in restraint and continued moving or struggling against the restraint. This movement was not usually detected by the telemetry system. Following the release from restraint, mice also showed marked increase in activity. They could be observed moving across the cage in various patterns (or no apparent pattern). Some mice involved in digging at times and more often they actively "explored" the cage. No formal attempt was taken

to analyse the behaviour of mice following the release after the tail-cuff protocol was complete. Activity level remained high, although highly variable, for approximately 40 minutes after the mice were released from restraint. After 45 minutes of activity the level decreased to zero and rarely rose to above 10 counts per minute.

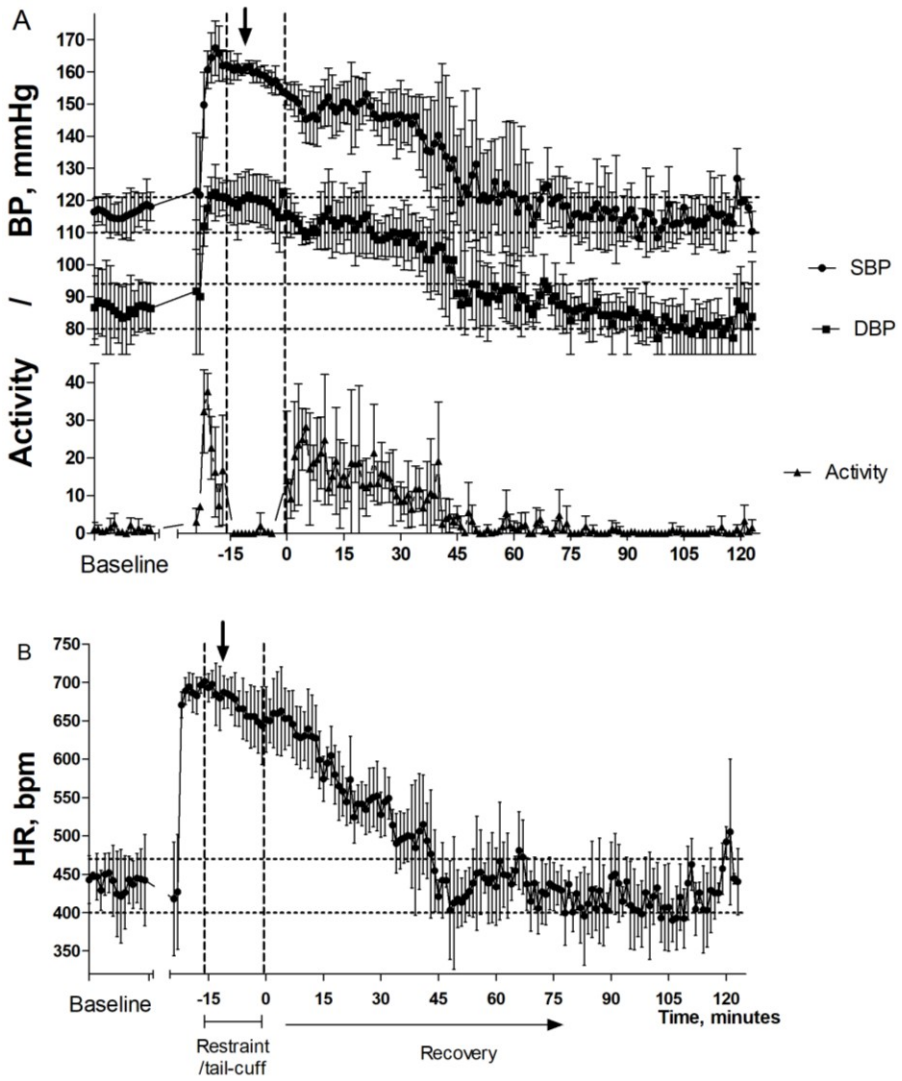


Figure 4.5. Haemodynamic profile during and until 2 hours after the tail-cuff recording: A, systolic (SBP) diastolic (DBP) blood pressure and activity; B, heart rate (HR). *Values are mean ± SD for 3 male C57Bl/6 mice, each data point represents 1 min average for 3 animals over 4 recording sessions to include the periods before the animals were disturbed on the day of each recording session (Baseline), when animals were picked up (at approximately -25 min time point) and placed into the restraint. The animals were in the restraint between -15 and 0 minutes (delimited by the broken vertical lines on the graph) and released to their home cages straight after. Dotted lines at 110…121mmHg, 80…94mmHg and 400…470 bmp delimit the approximate 75% range of blood pressure and heart rate most commonly observed during the "baseline" period.*

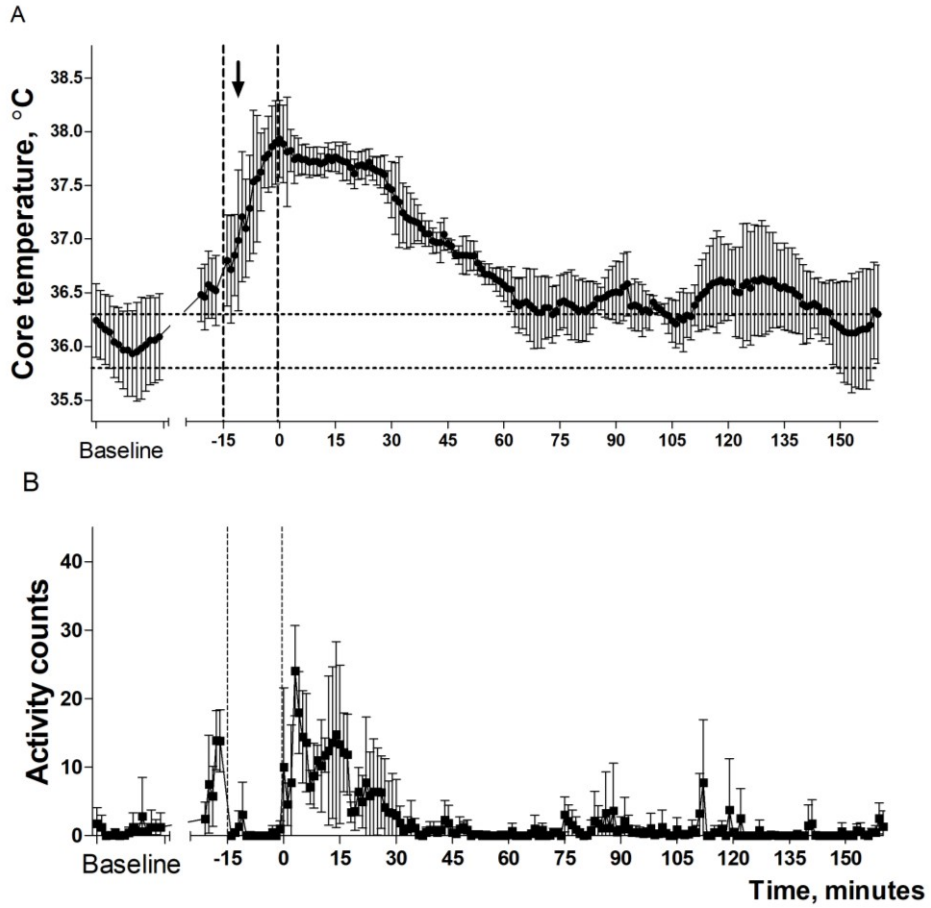


Figure 4.6. Changes in core body temperature before, during and after the tail-cuff protocol. Values are mean ± SD for 4 female mice subjected to the tail-cuff protocol on 3 occasions. Dotted horizontal lines represent upper and lower averages of the core body temperature during the baseline period. Vertical dashed lines between the -15 and 0 time points on the X-axis represent the start and finish of the tail-cuff protocol respectively

Core body temperature was 36.1°C on average and ranged between 35.6 and 36.7°C during the baseline period on the days the recording sessions took place (figure 4.6A). Mice were mainly at rest at that time with a few small outbursts of activity in some mice (figure 4.6B).

The core body temperature started rising during handling and continued so during the restraint, as already shown in figure 4.4. The recordings shown in figure 4.6 details the change in the measured parameters when the restraint period also included recording using the tail-cuff system (the start of the recording cycles is marked with arrow in both figures 4.6 and 4.4 for the relevant data set). The core body temperature started increasing once the mice were disturbed and handled for the tail-cuff protocol (before the -15 minute time-point on the X-axis) and it continued to rise steadily until it reached its peak value of 37.9±0.4°C after the 13-15th minute in restraint approximately.

Once the mice were released back to the home cages, core temperature plateaued at around 37.7±0.2°C for approximately 25 minutes. Thereafter the temperature decreased at a fairly

steady rate of 0.03° per minute for 40 minutes and after 1 hour after the tail-cuff protocol was finished, the core body temperature was 36.5±0.4°C. The temperature continued fluctuating around the same level for the hour and half that followed. Although this was at the higher end of the range, it did not significantly differ from the baseline readings taken before the tail-cuff sessions.

As also observed with mice implanted with telemetry devices (figure 4.5), activity increased sharply as soon as the mice were released back to their home cages. Although the activity tended to decrease, it remained elevated above zero for approximately 30 minutes. Thereafter the activity level mostly remained at zero mark with some occasional outbursts throughout the monitoring period.

The two groups of mice (implanted with cardiovascular and temperature telemetry probes) displayed similar activity patterns (as shown in figures 4.5B and 4.6B) and overall behaved similarly during and after the tail-cuff protocol. It was only female C57Bl6/J mice that were implanted with the temperature telemetry probes. I made the assumptions that the male mice display similar temperature changes during and following the tail-cuff protocol. Thus, it was decided not to implant male mice with temperature probes to explore if there are any gender differences.

4.3.4. Comparison of results obtained in male and female mice when subjected to the tail protocol.

I explored, however, how blood pressure and heart rate changes in male and female mice in response to the tail-cuff protocol. The cardiovascular parameters recorded by telemetry throughout the period that the tail-cuff protocol took place (including the baseline and recovery period available) were obtained under similar conditions in 9 male and 9 female mice on 2 occasions for each mouse were compared based on mouse's gender (figure 4.7).

All measured parameters before, during the restraint and after the mice were released to their home cages were remarkably similar between male and female mice. The only apparent difference was that the female mice had noticeably higher heart rate during the "baseline" period.

Blood pressure and heart rate increased in a similar manner in both genders when the mice were handled to be placed into the restraint. When mice were being placed into the restraint, the blood pressure peaked at 156.2±9.0/113.8±8.9 mmHg in male and 150.7±20.4/109.6±8.8 mmHg in female mice. The heart rate was 689±56 and 728±34 bpm in male and female mice respectively

during the same period. Halfway through the restraint period the blood pressure reached 159.3±17.0 /117.7±9.3 mmHg in female and 158.3±9.0/ 118.4±7.7 mmHg in the male mice, while the heart rate was 718±27 and 709±31 bpm in female and male mice respectively at the same time.

Following their release back to the home cages, the blood pressure did not appreciably decrease, and remained fairly level for approximately half an hour in both genders. Such that at 30 minutes following the release, the blood pressure was 149.7±26.7 / 111.5±21.7 mmHg in the females and 142.4±10.0 / 103.6±8.4 mmHg in the males, which is well within the standard deviation for the blood pressure for the last minute in restraint for the females and just about within the standard deviation for the males for the same period (the blood pressure was 150.1±17.2/106.9±7.2 and 152.6±7.6/114.4±7.2 mmHg during the last minute of restraint in females and males respectively).

The heart rate tended to gradually decrease similarly in male and female mice once they were released, in a way that is also similar to that shown in figure 4.5 for male mice only in a different group of male mice. During the first minute following the release it was 697±26 and 705±46 bpm in female and male mice respectively. Considering this period, i.e. the "recovery period", the number of female mice with the available recordings was gradually reducing shortly after the mice were released. Such, the recordings beyond the 20^{th} minute were only available for 3 female mice, compared to 9 male mice, and only for 2 female mice after the 40^{th} minute. This drop in female mice numbers most likely accounts for the fluctuation in the measured parameters for the female mice beyond the 20^{th} minute. I believe, however, that the data up to the 40^{th} minute for the female mice remains largely representative of the haemodynamic changes observed in the female mice in this study. Therefore, I shall not further interpret the data and conclude that male and female mice react similarly to the interventions associated with the tail-cuff protocol and, also, recover similarly after the tail-cuff protocol is complete. No significant difference in blood pressure, heart rate, or activity was detected between the male and female mice during the tail-cuff protocol or recovery.

The only difference between the groups, other than the difference in heart rate during the baseline period, was the variability of systolic blood pressure: the standard deviation observed in female mice was twice as large as the one observed in male mice.

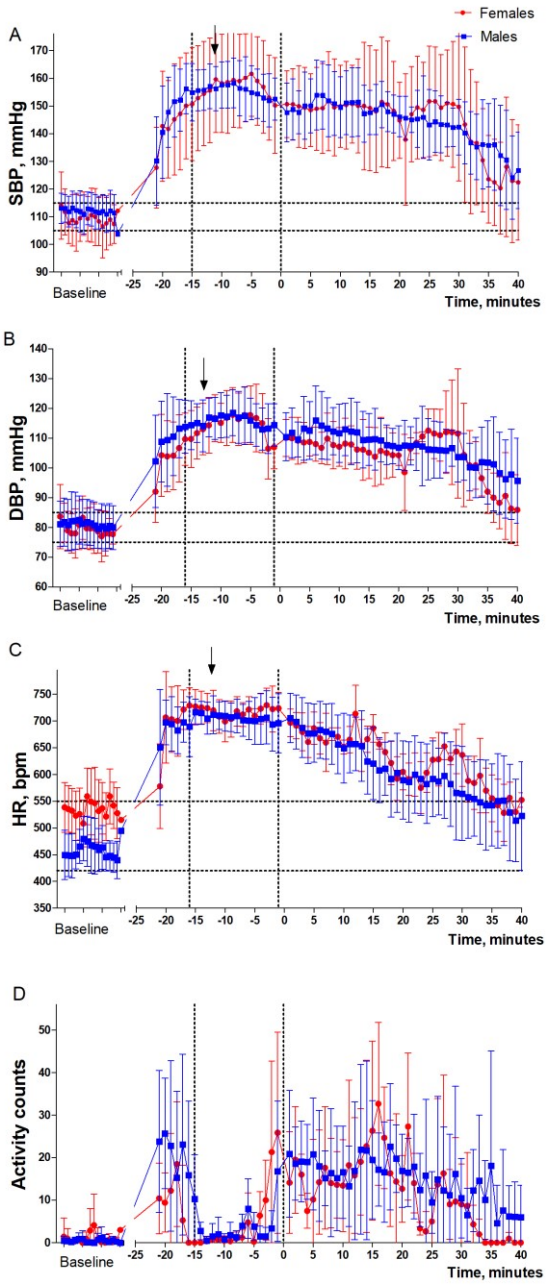


Figure 4.7. Haemodynamic and activity recordings throughout the tail-cuff protocol in male and female mice. *A) Systolic blood pressure (SBP), B) Diastolic blood pressure (DBP), C) Heart Rate (HR) and D) Activity counts in male and female mice before ("Baseline"), during the restraint and recording by the tail-cuff machine, and after the mice are released into the home cages. Scale on the X-axis represents the minutes following the release of the mouse back into the home cage. Mice were put into the restraint at -15 time point and the tail-cuff recording commenced at the -10th minute (shown with the arrow). Each data point represents Mean ± SD for 9 mice for each gender, over 3 recording sessions per each mouse.*

4.3.5. Analysis of the diurnal variation of blood pressure, temperature and activity in mice and influence on switching on telemetry switch.

The results in figure 4.8 show the diurnal variation of the cardiovascular parameters and activity in mice when they are undisturbed. The results show that the male and female mice had similar blood pressure profiles for both systolic, diastolic and pulse pressure throughout the light and

dark cycles. Such that the blood pressure during the light hours averaged around 114±8/82±6 mmHg and 118±9/87±7 mmHg in male and female mice respectively (Systolic / Diastolic blood pressure, Mean ± SD). During the dark, the blood pressure averaged around 132±13/96±9 and 137±9/101±6 mmHg in male and female mice respectively.

The heart rate was significantly higher in females during both day and night: it was 570±26 bpm in females versus 504±20 bpm in males during the light hours (***$p<0.001$); during the dark, the heart rate averaged at 623±31 bpm in females versus 579±14 bpm in males (**$p<0.01$). The heart rate is more variable during the night time, which most likely accounts for the smaller difference in the heart rate between the genders during these periods.

The average day-time activity was 3.3±1.7 and 3.4±1.7 counts per minute in male and female mice respectively. The nocturnal activity counts reached 23.0±11.8 in females versus 16.0±5.5 in the males. The females were very variable in the level of the night-time activity and the difference between the groups was not significant

All measured parameters varied significantly between the light and dark cycles, however only the heart rate varied significantly between the genders, with female mice having significantly higher average heart rate during both light and dark hours.

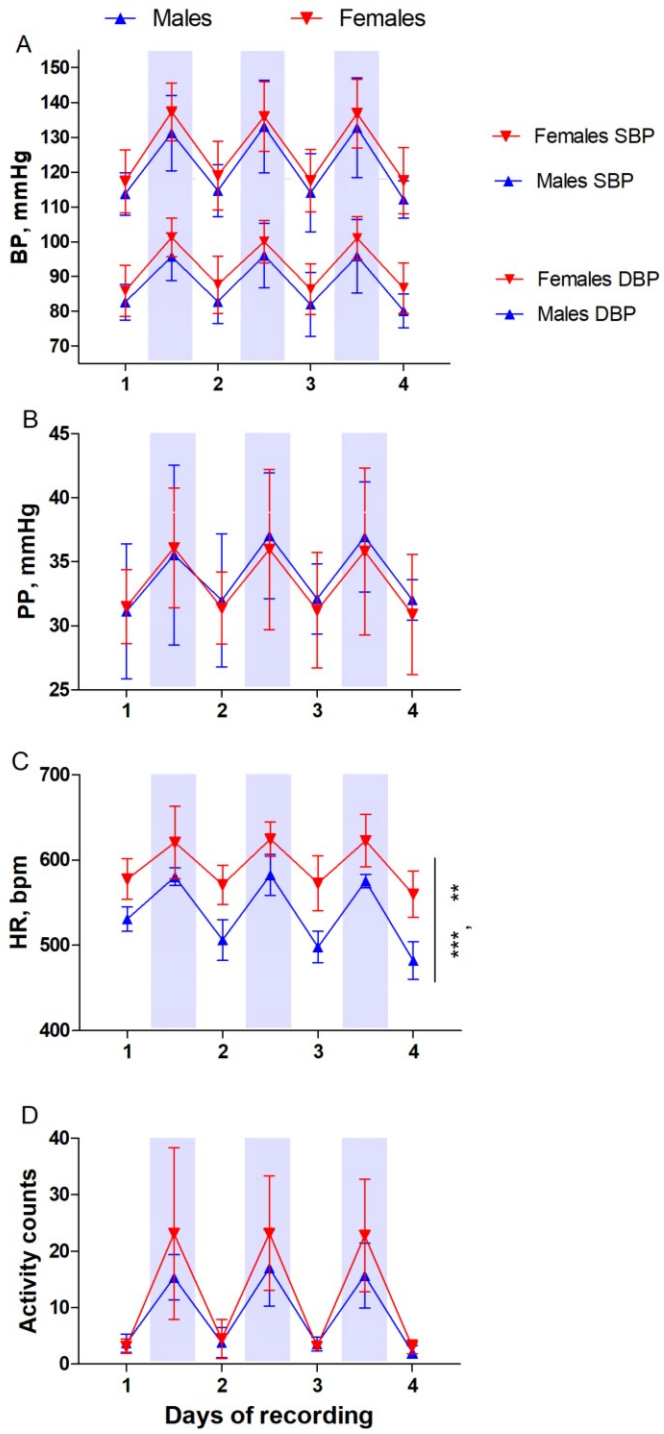


Figure 4.8. Baseline cardiovascular parameters (A-C) and activity (D) measured by telemetry in male (n=10) and female (n=11) C57Bl6/J mice. *A) Systolic and Diastolic Blood Pressure (SBP and DBP), B) Pulse Pressure (PP), C) Heart Rat (HR), D) Activity recorded by telemetry over 36 hours with minimal disturbance to the mice. While male and female mice have similar blood pressure, including pulse pressure (A-B), female mice have significantly higher heart rate (C, ***p<0.001) during both the light and the dark phases and higher activity during the dark phase (D, ***p<0.001). Two-way RM-ANOVA and Bonferroni post-tests. Each data point represents Mean ± SD for 10 male and 11 female C57Bl/6 mice.*

Temperature and activity were only measured in female mice, due to availability at the time. I decided not to extend the information to male mice as the body temperature was a secondary readout and published data is available for the genders in terms of diurnal variation.

Figure 4.9 shows diurnal variation in core body temperature and activity in female mice. Fluctuations in temperature and activity were significant between the light and dark phases ($p<0.0001$). During the light hours the average core body temperature was 36.4±0.2°C, while the night-time average temperature was approximately 1°C higher and was around 37.5±0.3°C. Day-time activity averaged at around 1.5±0.8 counts per minute, while night-time activity was 7.9±3.1 counts per minute on average. Interestingly, the female mice in this group had approximately 3 times lower night-time activity counts than the other group of females of the same strain (figure 4.8D).

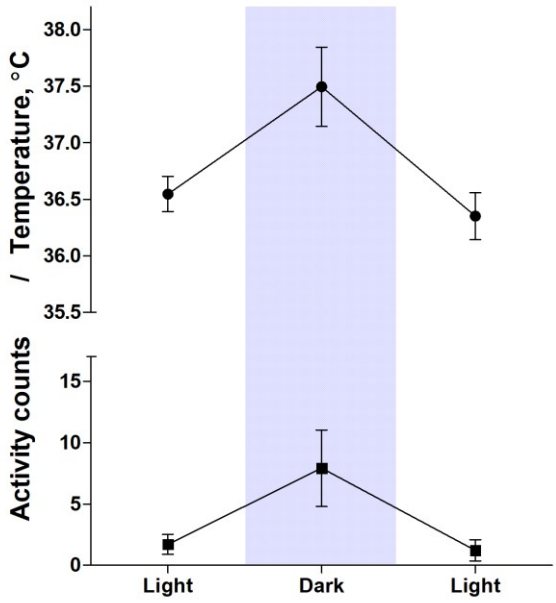


Figure 4.9. Core body temperature and activity measured by telemetry in female C57Bl6/J mice (n=4). Recorded over approximately 36-hour period in undisturbed mice. Each data point represents Mean ± SD for 4 female C57Bl6/J mice over the 12 hours of light or dark cycle as indicated.

It is worth bearing in mind that the values shown in figures 4.8 and 4.9 are averages for large data sets and, even though the error bars show standard deviation, the figures do not actually reflect the true variation of the measured parameters under the baseline conditions. The purpose of figures 4.10 - 4.12 that follow is to illustrate how the core body temperature and haemodynamic parameters vary during the dark and light hours. I also included these variables as measured during the restraint period of the tail-cuff protocol to illustrate how these compare to the range of values obtained when mice were not known to be disturbed.

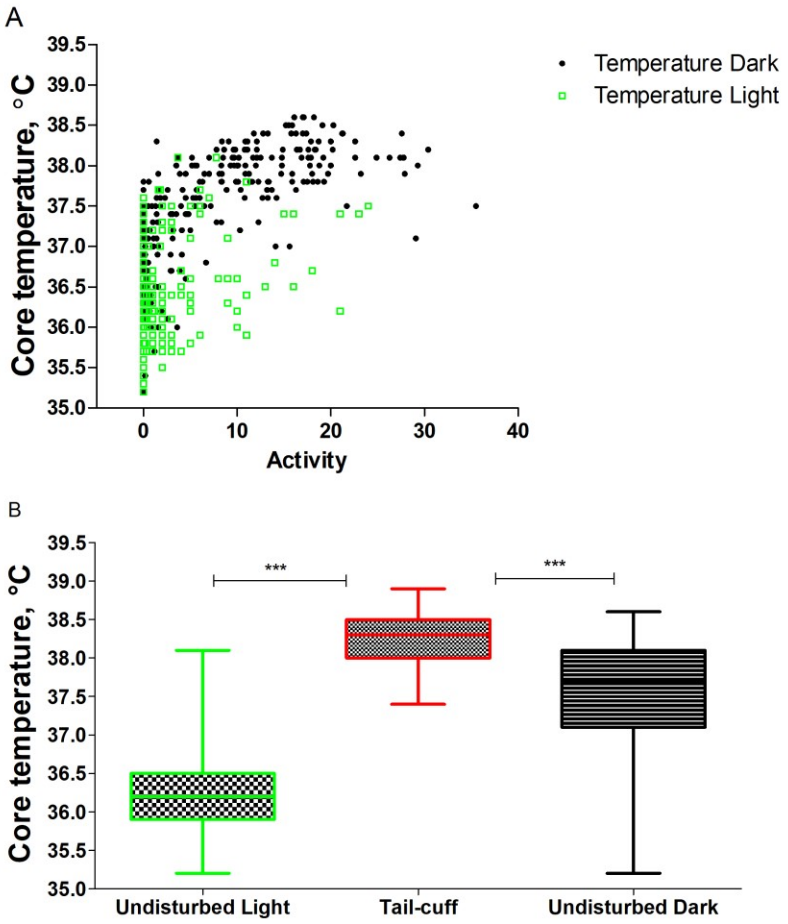


Figure 4.10. Correlation of activity and core body temperature (A) and range of core body temperature measurements during recording by the tail-cuff technique and during the light and dark hours when the mice were presumed to be undisturbed (B). *Each data point represents an individual measurement by telemetry taken in 10-second bins (Tail-cuff), or 1, or 10-minute bins (Undisturbed light and dark) in one mouse, for 4 female C57Bl6/J mice in total. There are 236 data points to represent the measured parameters during the dark hours (A and B), three are approximately 760 data points to represent the measured parameters during the light hours and there are approximately 400 data points to represent the temperature measurements during the tail-cuff protocol (B).*

Core body temperature rises with activity. The correlation analysis of the two parameters (figure 4.10A) has not been performed because this type of analysis does not take into account the influence of the previous period. The different rates of change in each of the measured parameter would make the analysis misleading. However, I find this is a convenient way to show the two parameters in the same graph. Core body temperature is typically lower when the mice are at rest and rises when they become active. Although the range of core body temperatures at zero activity over the same period was 35.2 to 37.8°C. When a higher temperature is observed during the period of no activity, this period is typically preceded by periods with activity. Small activity spikes, such as under 5 counts, may or may not entail increase in core body temperature. The prevalent recording schedule of 10 second recording every 10 minutes during the overall baseline

recording period (that was taken over the 36 hours) did not allow me to resolve if the core temperature can rise in anticipation of activity.

The maximum temperature achieved during spontaneous activity was 38.6°C (at night), while the activity counts at the same time ranged between 16 and 18 counts per minute. The period of maximum activity, as shown in the Fig 4.10A, is 35.5 counts per minute, with an accompanying body temperature of 37.5°C, which continued to rise and reached 38.1°C in the next 10 minutes when the activity was maintained, albeit at lower level (this was also during night time).

Day-time period, which is typically when the mice are less active, is characterised by lower activity and lower body temperature range, with most data contained within the 35.9°C and 36.5° core body temperature range (mean value ± SD were 36.2±0.4°C). During the night, the activity and core body temperature were typically higher. The core body temperature had wider distribution and the inter-quartile range was between 37.1 and 38.1°C, with the mean value 37.5±7°C. The tail-cuff protocol took place during the day and it caused significant increases compared to the preceding resting period used for comparison. The interquartile range of core temperature during the tail-cuff protocol measured between 38.0 and 38.5°C, while the mean temperature was 38.2±0.3°C. Although the overlap with the night-time period was substantial, the difference between the temperatures measured during the night and the tail-cuff protocol was still significant (***$p<0.001$).

Blood pressure and heart rate (figures 4.11 and 4.12) also ranged greatly within each period. Approximately 50% of the systolic blood pressure measurements for the females ranged between 100 and 123mmHg and for the males between 104 and 130mmHg during day; and in the absence of any known disturbances, some values reached over 170mmHg for male mice and 163mmHg in female mice. The maximum value for the heart rate was 808bpm for the male mice and 738bpm for the female mice. As far as the interquartile range is concerned, the heart rate ranged between 527 and 631 bpm in the female mice and 455 and 577 bpm in the male mice. The total range of heart rate measurements in the male mice was larger than that of the females, especially at the lower end.

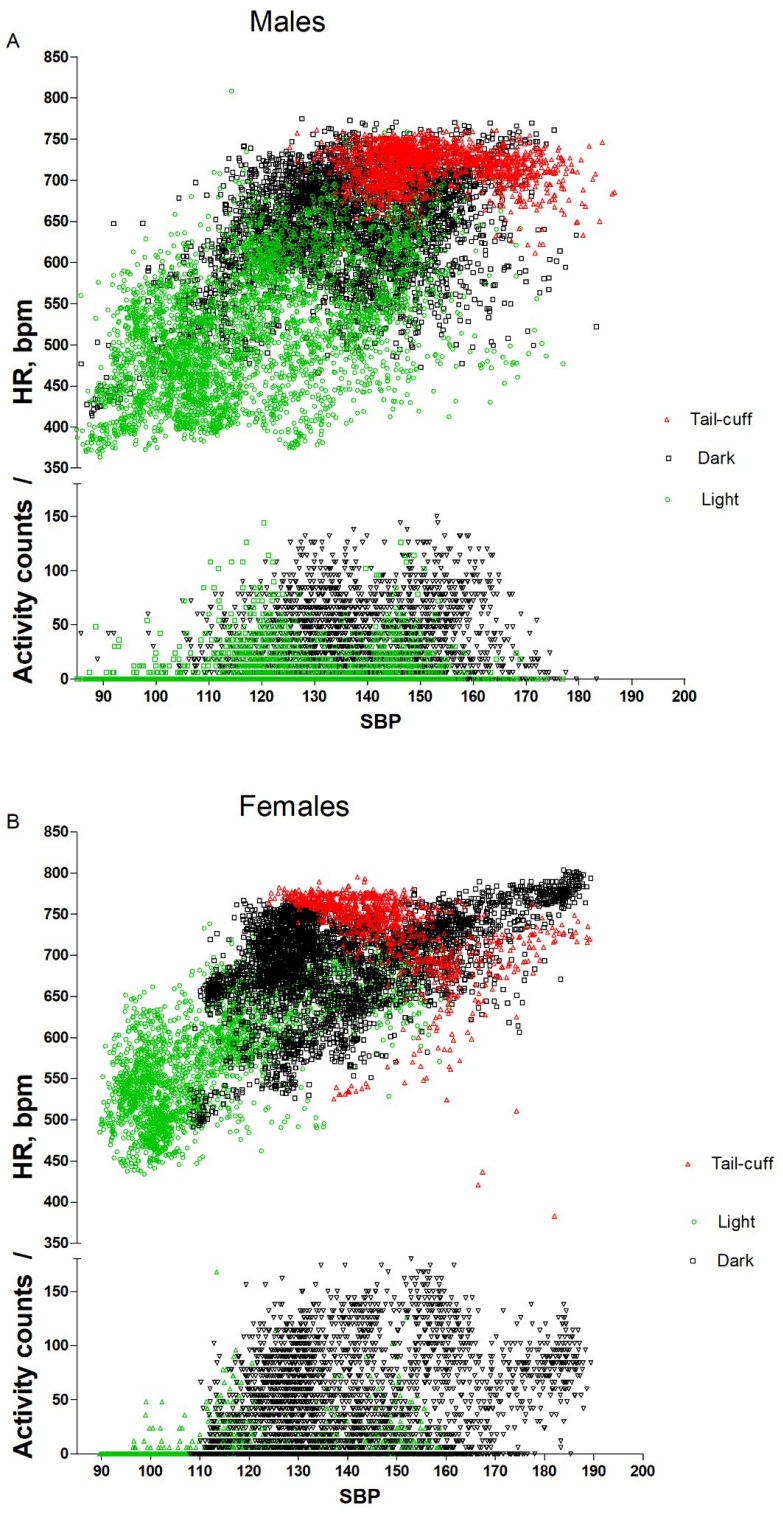


Figure 4.11. Correlation of activity heart rate and blood pressure and blood pressure and activity in the undisturbed free moving mice in their home cages during the light or dark hours and during the recording by the tail-cuff technique male (A) and female (B) mice. *Each data point represents individual measurements by telemetry taken in 10-second bins (Tail-cuff), or 10-secod, 1, or 10-minute bins (Undisturbed light and dark) in one mouse, for 6 male and 6 female C57Bl6/J mice in total. Each data set includes approximately 3600 data points for the measured parameters (600 for each mouse) for undisturbed and approximately 200 data points per mouse for the tail-cuff period.*

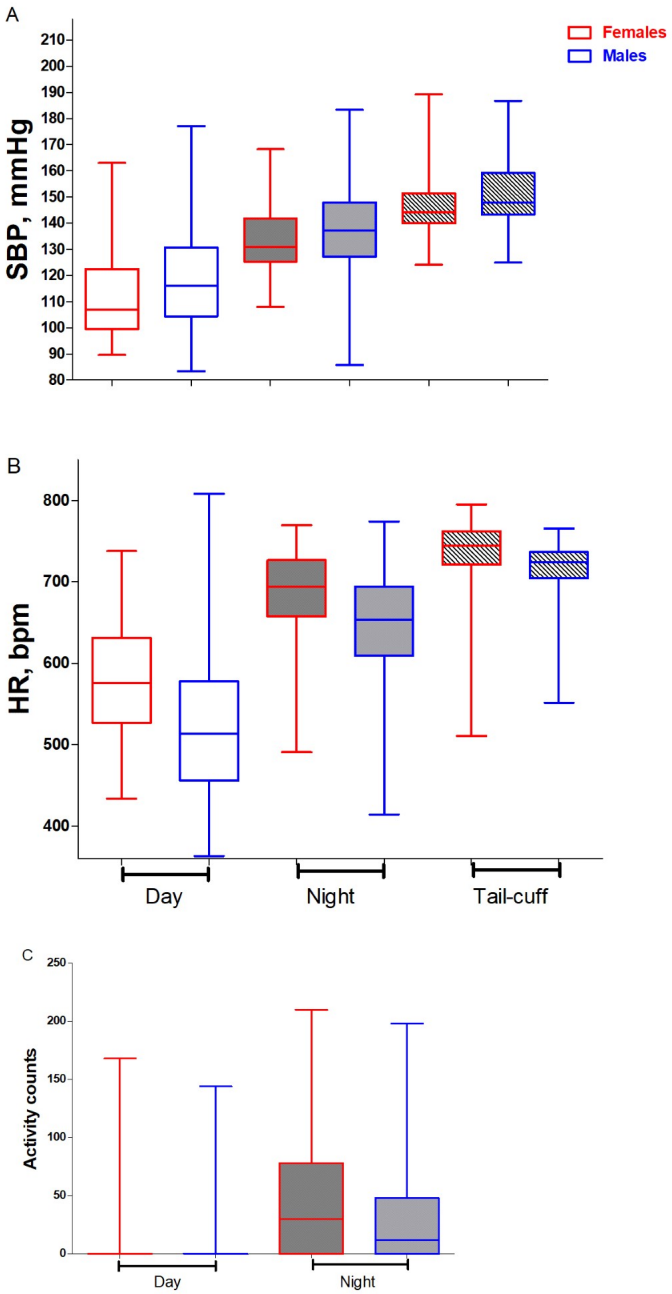


Figure 4.12. Systolic blood pressure (A), heart rate (B) and activity (C) in the undisturbed free moving male and female mice in their home cages during the light or dark hours and during the recording by the tail-cuff technique. *Each data set is shown as a "box and whisker" plot that includes approximately 3600 data points for each of the measured parameters in 6 male and 6 female C57Bl6/J mice in total (600 for each mouse) for undisturbed and approximately 200 data points per mouse for the tail-cuff period. These measurements are taken in 10-second bins (during the Tail-cuff), or 10-secod, 1, or 10-minute bins (during the Day and Night in undisturbed free-moving mice).*

The night time period was characterised by higher activity, blood pressure and heart rate for both male and female mice, as already shown in figures 4.8A-D. Figure 4.12 further demonstrates that 50% of measurements of systolic blood pressure ranged between 125 and 142mmHg in the females and 127 and 148mmHg in the males. The systolic blood pressure exceeded 165mmHg in the females and 180mmHg in male and female mice on some occasions, typically during the

periods of high activity at night (figure 4.11). The interquartile range for heart rate was between 660 and 734bpm in female mice and 610 and 695 bpm in the male mice during the night. The systolic blood pressure and heart rate measurements taken during the tail-cuff protocol overlapped considerably with the measurements taken during the night hours for male mice. Such, the interquartile range for systolic blood pressure while the mice were in restraint was 140 -160mmHg and 140 - 151mmHg for male and female mice respectively, and the heart rate range was 720 and 760bpm for the female mice and 705 and 737bpm for the male mice.

The maximum values for the systolic blood pressure and heart rate during the night were: 168mmHg and 769bpm in female, 183mmHg and 774bpm in the male mice. The maximum values achieved during the tail-cuff protocol were 189mmHg and 795bpm for the female and 186mmHg and 765bpm in the male mice.

Although there is a large overlap in the magnitudes obtained for the measured cardiovascular parameters in the restrained and freely moving mice, the tail-cuff protocol induced maximal increases in the cardiovascular parameters that nevertheless could also be observed in the free-moving mice. Over 50% of the systolic blood pressure taken during the restraint fell above the median value for the respective readings obtained during the dark hours when the mice were more active. The vast majority of the heart rate measurements obtained during the tail-cuff protocol lied within or just above the upper quartile of the respective measurements obtained in freely moving mice during the period of high activity.

4.3.6. Effect of switching on of the telemetry probes on haemodynamic parameters and core body temperature.

It was shown earlier (figures 4.1 and 4.2) that a brief human presence (researcher entering the room) does not significantly affect the haemodynamics, activity or core body temperature compared to the similar period during the day when there is no human present in the room. However, it was observed that switching on the telemetry probes, does appear to induce noticeable changes in the measured parameters and I decided to quantify these changes and how long these potentially lasted. To activate the probes, the researcher must enter the room, approach the cages in order to pass the magnet close to the animal to switch on the probes. This may or may not involve moving the cage, making a noise, etc. Recordings not confounded by other interventions and within a comparable time during the day were available for most mice on at least one occasion. This data is presented in figures 4.13 for cardiovascular and activity, and figure 4.14 for temperature and activity.

Figure 4.13 below shows that switching on of telemetry probes impacts all the measured parameters for approximately 25 minutes. The probes were switched on in the daytime, when they were typically in their nests resting, thus, switching on the probes acted as a disturbance. However, there was large variability associated with switching on the probes. Two-way RM-ANOVA could not be performed using the available GraphPad prism software version 5. Alternatively, One-way ANOVA test was performed instead to compare the genders and the time periods to include the first 20 minutes and 25- 45 minutes after the probes were switched on. There was no difference between the genders for either period, however there was significant difference between the first 20 minutes and 25-45 minutes for systolic, diastolic blood pressure and heart rate both genders (*$p<0.005$) Switching of the probes apparently causes approximate 15 mmHg and 50 beats per minute increase in the heart rate. This may be a considerable increase for some experiments.

Body temperature started rising from the first minute after the probes were on (and thus the recording could take place, figure 4.14). The core temperature peaked at 37.4±0.3°C between the 10^{th} and 14^{th} minute of the recording and returned to a stable "baseline" after approximately 30 – 40 min. Interestingly, the variability of temperature measurements between the 10^{th} and the 14^{th} minutes was lower than that observed for the period that followed. No statistical analysis was carried out on this data.

Increases in both the temperature and cardiovascular parameters were associated with the increases in activity. The increases in activity observed in these groups of mice were comparable (figures 4.13 A and 4.14).

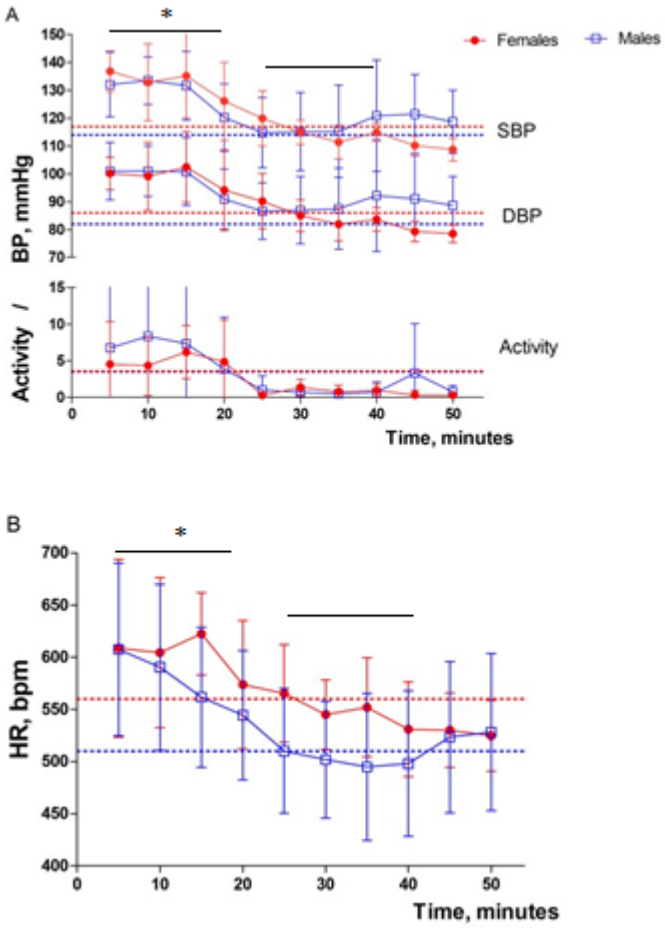


Figure 4.13. Changes in haemodynamics and activity in C57Bl6/J male and female mice from the time-point the telemetry devices were switched on. *Changes in systolic (SDP), diastolic (DBP) blood pressure, activity (A), and (B) heart rate (HR) in male (n=10, blue solid line) and female (n=7, red solid line). Each data point represents Mean ± SD for 10 male or 7 female mice for one recording session. The blue or red dotted lines represent the reference mean values for males or females (respectively) for systolic, diastolic blood pressure, activity (A), and heart rate (B).One-way ANOVA was used to compare the two periods defined by a solid line, *p<0.005 denotes the difference between the two periods for each gender.*

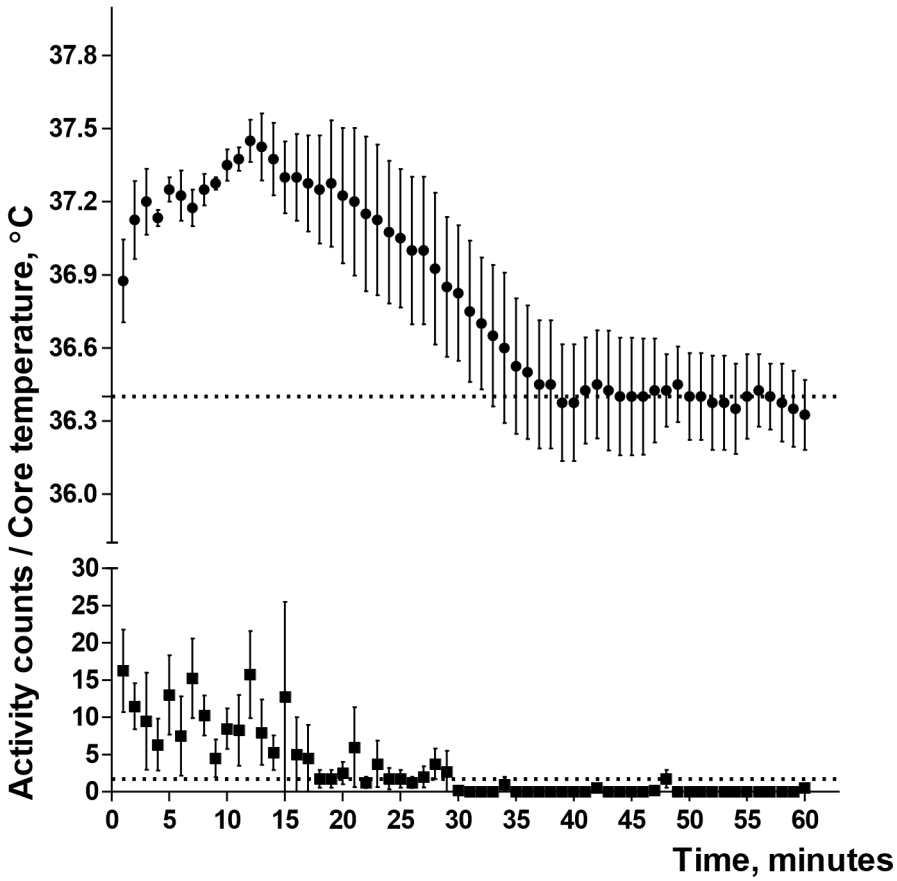


Figure 4.14. Changes in core body temperature and activity in C57Bl6/J female mice (n=4) from the timepoint the implanted telemetry devices are switched on. *Each data point represents mean ± SD value for 4 female mice obtained on one occasion. Dotted lines represent an estimated mean core body temperature and activity levels in undisturbed mice at rest.*

The next question I asked was whether this disturbance after switching on the probes was always observed, or whether there was an element of habituation. I analysed the available recordings if the mice respond with lower increases in the measured parameters on subsequent occasions when the telemetry probes were switched on. Figures 4.13 and 4.14 show that the maximum increase in haemodynamics or core body temperature are achieved within 15 minutes of switching the probes. To compare the maximum increases achieved on each occasion, I calculated the average value for each parameter over the 15 minutes after the probes were switched on. The consecutive days of recording for cardiovascular and core temperature data, separated by some days when no recordings were made as specified, are presented in figures 4.15 and 4.16.

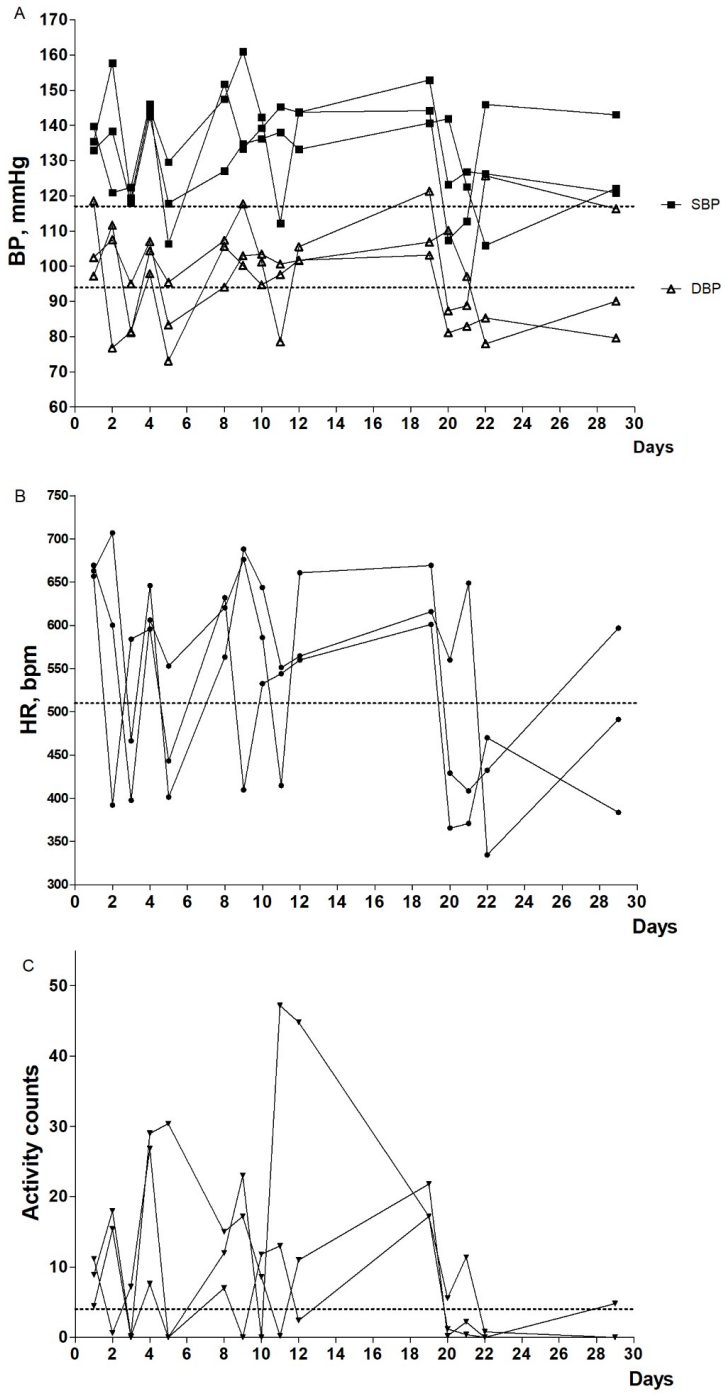


Figure 4.15 Blood pressure and heart rate and activity during the first 15 minutes after the telemetry transmitters are activated. *A) Systolic (SBP) and diastolic (DBP) blood pressure; B) heart rate (HR); C) Activity counts. Each data point represents the average value for the first 15 minutes following the activation of the telemetry probe on each day of the recording for one mouse. The graph shows the data for three male C57Bl6/J mice over 29 days of recording. Dotted lines represent estimated average resting blood pressure and activity.*

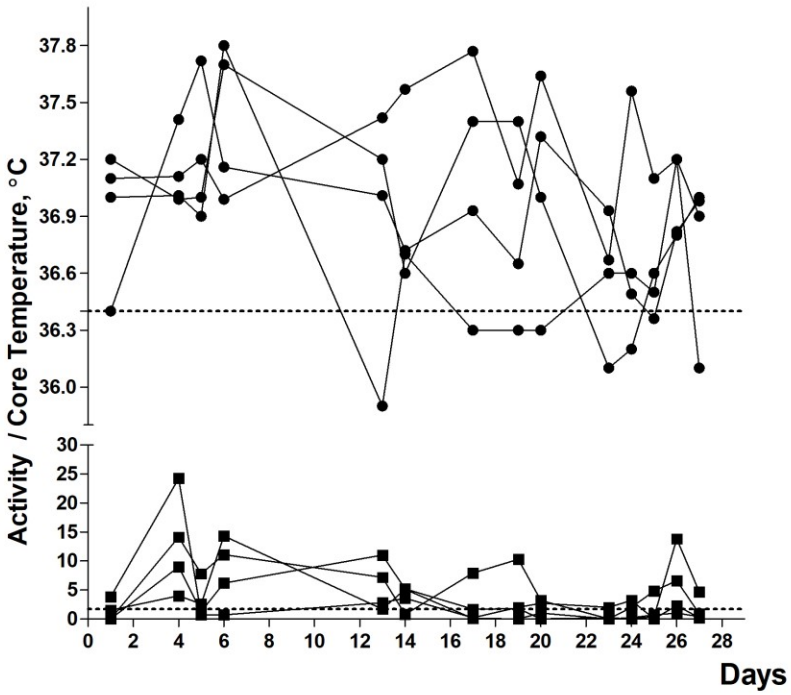


Figure 4.16. Core body temperature and activity during the first 15 minutes after the telemetry transmitters are activated. Each data point represents the average value the first 15 minutes following the activation of the telemetry probe on each day of the recording for one mouse. The graph shows the data for four female C57Bl6/J mice over 27 days of recording. Dashed lines represent estimated average resting core body temperature and activity.

High variability was observed for all the mice and therefore I decided to show this data as individual data points obtained for each mouse on each occasion. There is very high variability between the mice and between the days for the same mouse. This leads me to conclude that there is no habituation to this intervention as such and the impact of other factors is significant and predominant.

4.3.7. Do the mice become habituated to the tail cuff protocol

The final set of results in this section was from analysis of data from previous studies to determine whether the mice became habituated to the tail cuff technique. The tail cuff technique was carried out over a month on the days indicated. The results of measuring blood pressure and heart rate by telemetry indicate that the mice do not become habituated. The blood pressure and heart rate measurements obtained on the first day were 162±9/122±14 mmHg and 699±16bpm (Mean ± SD). Although the measurement on the 5th day were somewhat lower, 153±6/116±9 mmHg blood pressure and 691±40 bpm heart rate, this trend did not continue, nor repeat during the two further rounds of 5 consecutive day recordings.

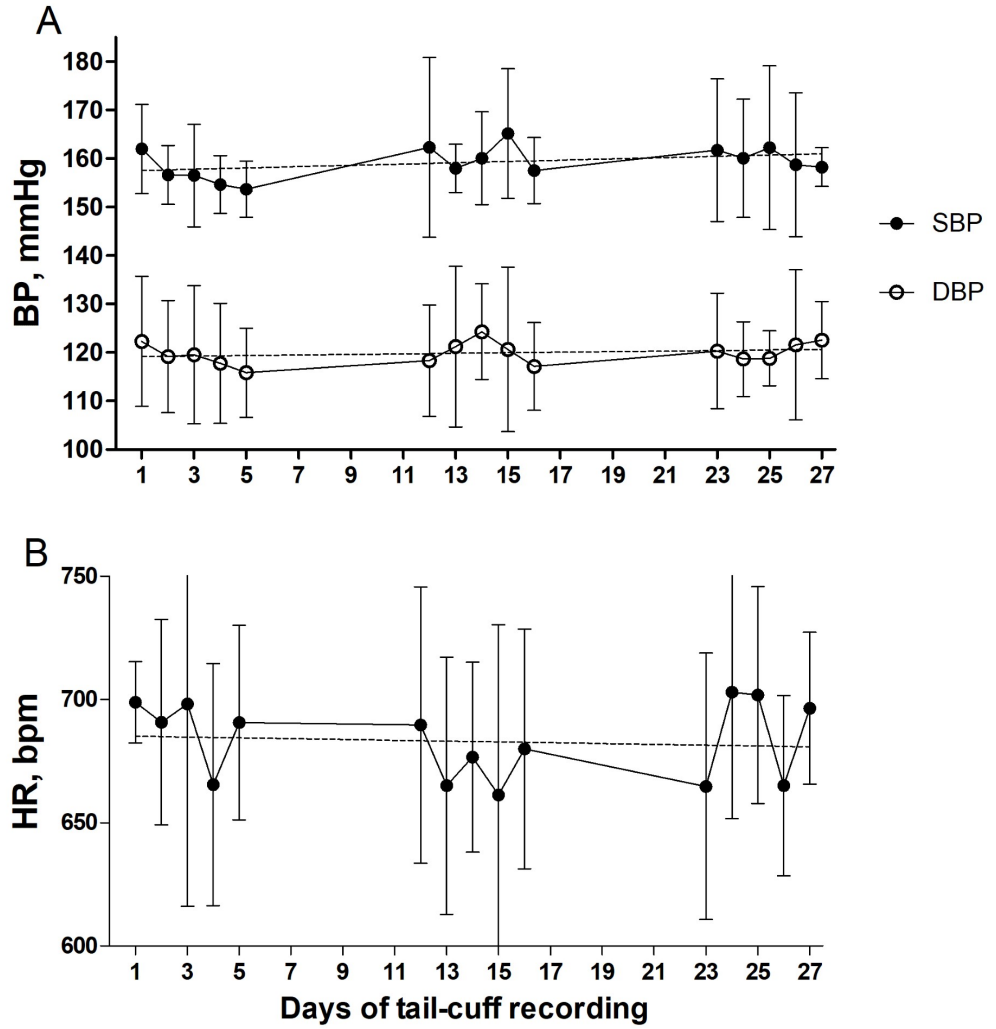


Figure 4.17 Telemetry recordings of cardiovascular parameters during repeated exposures to the tail-cuff technique. *A) Systolic (SBP) and diastolic (DBP) blood pressure, and B) heart rate (HR). The results are from a study where the mice were exposed to the tail-cuff technique on days 1-5, 12-16 and 23-28. Values are mean ± SD for 6 male C57Bl6/J mice. The dotted line represent the Linear Regression Analysis between the recorded parameters (blood pressure, A and heart rate, B) and the days of the tail-cuff recording.*

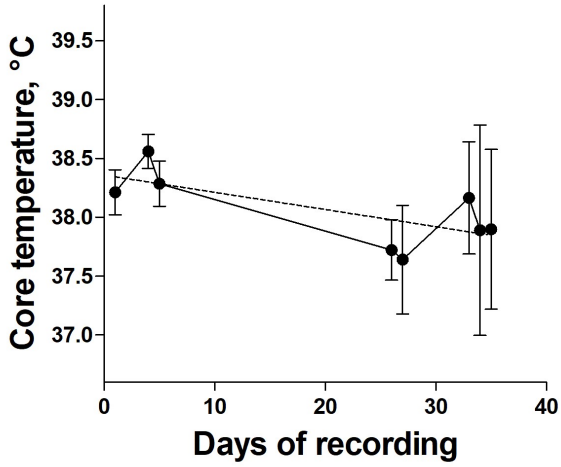


Figure 4.18. Telemetry recordings of core body temperature during repeated restraint for the tail-cuff technique. *The results are from a study where the mice were restrained with pre-heating on days 1-3, 27-28 and 3335, where day 1 is the 1st day of tail-cuff recording at least 10 days after telemetry device was implanted. Values are mean ± SD for the last 5 minutes of restraint for 4 female C57Bl6/J mice. Dashed line represents regression slope which is significantly different form zero (p=0.0248) between core body temperature and days of the recording.*

Figure 4.18 shows the mean over the last 5 minutes when the mice were in the restraint in the tubes that had been pre-heated and their blood pressure was recorded using the tail-cuff technique over the days of recording as indicated on the X-axis. The mice were restrained in the tubes without pre-heating on the days between 10 and 20, however this data has not been shown here. The core body temperature reached its peak during the last 5 minutes (out of typically 15 minutes) of restraint, therefore this read-out was chosen to reflect the maximum physiological effect and a stress marker. The core body temperature measurements taken during the last 5 minutes shows a trend to decrease over time. As indicated by the linear regression analysis, this trend appears to be significant (p=0.0248), however the difference between the first (days 1, 4 and 5) and last (days 33, 34, 35) three days was not significant (p=0.1525).

The cardiovascular parameters, as measured by telemetry during the tail-cuff protocol, remained to be similarly elevated at the beginning and the end of the experimental period. This suggests that the mice do not habituate to the tail-cuff protocol over time. The core body temperature, on the other hand, appeared to be lower following a number of restraint sessions. Furthermore, most mice used in these experiments appeared to become habituated to the user and to the process involving being placed in the tube became quicker over time. Perhaps the relatively lower increase in the core body temperature indicates there is a degree habituation to handling and restraint, even though this is not translated into a meaningful reduction in the measured haemodynamic parameters.

4.4. Discussion

The main outcomes of this chapter are that the interventions that contain restraint induce high and sustained changes in blood pressure, heart rate and core temperature. However, whether the restraint also involves heating and further tail cuff occlusion cycles, did not make any significant difference to the measured haemodynamic parameters. This rise in all the measured parameters is maintained throughout the restraint period and there is no subsequent decrease in these responses on repeated exposures.

Restraint without pre-heating the tubes caused a dip in core body temperature from the first minute for at least 5 minutes, after which point the temperature progressively increased. This is believed to be due the cooling effect of the tube that was at ambient temperature and this is below thermoneutral range for the mouse (Swoap et al., 2004, 2008)

Handling caused a very sharp rise in blood pressure and heart rate, that was identical to the restraint period, however this increase dissipated fairly rapidly after the handling was complete. The effect of handling on the core body temperature was however sustained throughout the measured 15-minute period and was indistinguishable from the tail-cuff and restraint in preheated tube interventions. Moving the cages also caused significant perturbation in all the measured parameters: heart rate response in the first 2 minutes was almost indistinguishable from the response to restraint, however blood pressure and temperature increases were lower. “Entering the room” intervention caused no appreciable effects on the core body temperature or haemodynamics.

Telemetry-based experiments on the effect of handling on the blood pressure during the tail-cuff protocol (described in chapter 3, see figure 3.17) suggest that there is no apparent reduction in blood pressure or heart rate response to repeated handling. Figures 4.17 and 4.18 similarly show that there was no reduction in the measured cardiovascular parameters and core body temperature in response to repeated tail-cuff protocol.

The rationale behind the interventions identified for this study was to use a step-wise increase in the stressing factor that these interventions would entail: from the hypothetically minimal “entering the room” to the most distressing “tail-cuff” intervention that contains cage movement, handling, restraint, warming the mouse and constricting the tail. The maximum increase in blood pressure was achieved during restraint, without further increases achieved when heating and tail constrictions were added. The latter two interventions were not tested in isolation from restraint.

Swoap and colleagues (2004, 2008) studied the temperature range between 18 and 30°C and their conclusion was temperature range 29 - 30°C was within thermoneutral zone for mice, who had approximately 10mmHg reduction in mean arterial pressure and at least 50 bpm reduction in the heart rate at that temperature compared to 22-24°C. Based on unpublished observations made during these experiments, I do not believe that warming mice to 35°-35°C is likely to be noxious for mice, and, based on the results shown here, does not led to further increase or decrease in the haemodynamic parameters. Any effects were likely overridden by the impact of restraint. Stress hormone levels were not tested in these studies because it was believed that these measurements would further affect the primary biomarker, i.e. the blood pressure. No assay to measure the impact of tail constrictions without restraint or handling was readily available. Because the restraint is not possible to eliminate from the tail-cuff protocol for mice, the impact of tail constrictions without restraint was not pursued to explore.

Both cardiovascular parameters and core body temperature change dramatically in response to a number of stimuli and are regulated by a range of internal and external factors. I observed that free-moving female mice (in their home cages) have higher heart rate during the day and at night compared to the males (Figure 4.8), yet there was no difference in blood pressure. Despite the understood gender differences in regulation of the cardiovascular system (Scotland et al., 2005; Maranon and Reckelhoff, 2013; Ji et al., 2014) and body temperature (Sanchez-Alavez et al., 2011), both genders reacted similarly in terms of the heart rate and blood pressure changes in response to all the identified interventions, starting from the least intrusive switching on the telemetry probes to the restraint and the tail-cuff protocol.

The blood pressure and heart rate changed almost undistinguishably in males and females before, during, and after the tail-cuff protocol. The only difference was that the female mice had larger variation in systolic blood pressure (but not other parameters). It is not clear if this is an effect of oestrus cycle that particularly affect the peak of the pressure wave.

Blood pressure, heart rate and core body temperature returned to the resting level approximately 1 hour after the tail-cuff protocol was complete. The tail-cuff protocol was conducted in one mouse at a time, typically 3 or 5 mice in a day. Continued human presence and the noise from mouse cage movement most likely affected the rate and the extent how the measured parameters changed for each mouse depending on the order in the testing sequence that each mouse had. Although vocalisation by mice was rare during the protocol, human ear is not equipped to perceive the sounds heard and communicated by mice. This, as well as other stimuli that humans cannot perceive, can be the unknown variables that may have an impact.

The apparently larger effect of handling on core body temperature compared to moving the cage, may be the manifestation that handling is perceived as considerably more noxious than mere cage movement. All the measured parameters following handling typically returned to baseline between half an hour and an hour and more like within 30 minutes following cage movement.

Turning on the telemetry probes also involves an element of disturbance to mice that may disturb haemodynamics and core body temperature for up to 30 minutes. However, this did not always happen, nor that there was an apparent trend for change over time. It does not appear that this effect was due to the recording artefacts, and it appears to me it was most likely due to the variable amount of noise and other disturbance to the mice for the researcher to reach the probes.

Likewise, mice did not appear to habituate to the tail-cuff protocol as there was no apparent reduction in blood pressure, heart rate (and most likely not core body temperature) that the tail-cuff otherwise caused. When I carried out this study, I thought the results with habituation were some of the most important findings of my thesis and, to my knowledge, novel. However, we now realise that Sikora and colleagues (Sikora et al., 2016) published a manuscript with similarities to our own research in the same year (2016), as our own was published. They studied the effect of restraint stress in rats on blood pressure, as measured by telemetry, over 60 minutes. Their results agree with this study, in that restraint increased blood pressure and no habituation was observed. They discussed the established concept that five days of training is suggested before experimental measurements for the tail cuff and that some suggest longer (e.g. Kurtz et al. suggest 14 days). However, they, like me, suggest that this does not influence the effect of restraint stress on blood pressure. They also cite another paper, also referenced in our publication, where 10 days of training had no influence in mice (Gross and Luft, 2003).

To conclude; results in this chapter have allowed me to provide evidence that a range of interventions considerably disturb the mouse. The restraint step of the tail cuff method produces large changes in cardiovascular parameters that are largely not realised from the measurement of the tail blood pressure

Chapter 5.

Comparison of Recordings Made by the Tail-Cuff and Telemetry

5.1. Introduction

Non-invasive indirect techniques to measure blood pressure have been compared to the direct methods since the very early days when the non-invasive techniques first came about. This was achieved in the same (anaesthetised) mouse (Bonsmann, 1934; Byrom and Wilson, 1938), or relied on other studies that used conscious or anaesthetised animals (Williams et al., 1939). Telemetry technology for blood pressure measurement in small animals facilitated this comparison and made it possible that blood pressure can be measured in conscious animals by the two techniques simultaneously (Whitesall et al., 2004; Feng et al., 2008). It is generally believed that the tail artery in the mouse or the rat should have the same pressure as a central artery and the VPR tail-cuff system, also used in this work, is able to estimate central blood pressure in conscious mice with minimal difference between the two techniques (Feng et al., 2008). When I used telemetry to measure blood pressure in the mouse during handling and the tail-cuff protocol, I observed that the measurements provided by the two technique were markedly different for both systolic and diastolic blood pressure

The main aim of this chapter is to compare the recordings obtained by the telemetry and the tail-cuff in the same mouse at the same time. These measurements will be referred to as "simultaneous" recordings. Because telemetry-based studies allow blood pressure recordings made in undisturbed mice, I think it will be important to include the recordings obtained by telemetry in the undisturbed mice and also compare those to the recordings obtained by the tail-cuff. These readings are obtained in the same mouse, but not at the same time and will be referred to as "non-simultaneous" measurements.

Volume Pressure Recording (VPR) sensor technology for tail-cuff used in this study and presently one of the most widely used, was validated by Feng et al (Feng et al., 2008) relying on Bland-Altman analysis that checks the agreement between the techniques. It is commonly cited that they found negligible difference between telemetry and the VPR systems (Savas et al., 2016; Wang et al., 2016), despite an apparently large difference in individual recordings or lack of consistent results across the whole range of blood pressure recordings. I shall use both correlation analysis and the Bland Altman plot that is otherwise recommended to test agreement between the different measurement techniques (Bland and Altman, 1986; McLaughlin, 2013).

The goal of this study is to compare and correlate the recordings obtained by telemetry and the tail-cuff techniques in the same mouse. Aims were:

- To compare and correlate tail-cuff and telemetry recordings made in the same mouse at the same time.
- To compare and correlate telemetry recordings made in undisturbed mouse and the corresponding tail-cuff recordings made in the same mouse on the same day
- To compare the two techniques in measuring blood pressure in normotensive and hypertensive mice following Ang II infusion (one of the most common hypertension models).

5.2. Protocol details including experimental design and development

5.2.1. Animals

The experiments included in this chapter were carried out in male (n=13) and female (n=11) C57Bl6/J mice implanted with blood pressure telemetry (PA-C10, DSI) probes. Age range of mice used in these experiments ranged between 12 -15 weeks at the start of the experiments. The data presented in this chapter focuses on the correlation and comparison of the two techniques to measure blood pressure in the mouse (tail-cuff and telemetry) and includes the results obtained in 5 groups of C57Bl6/J mice (13 males and 11 females) implanted with the blood pressure telemetry probes at different time-points of this project.

5.2.2. Experimental design

All the details of surgery and the procedures for blood pressure recordings made by telemetry and the tail-cuff, including the simultaneous and non-simultaneous recordings by the two techniques can be found in chapter 2.

Table 5.1 Overview of studies included in this chapter.

Chronological order	1	2	3	4	5
Number and gender of mice	*3 males*	4 males	5 females	4 males + 4 females	2 males + 2 females
Hypertension (Ang-II) model?	*No*	No	No	Yes	Yes

Studies number 1 - 3 include only the data obtained in mice that were not made hypertensive using the Ang-II infusion. Studies 1 and 2 were the handling studies. Since the different handling techniques induced very similar cardiovascular responses during handling and later during the restraint and recording by the tail-cuff technique and this session were pooled together and included in the analyses to compare the tail-cuff and telemetry systems.

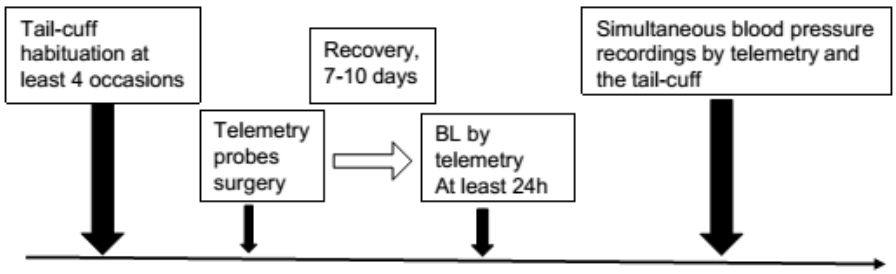


Diagram 5.1. The experimental procedures and their timings as applicable to studies number 1 - 3. BL = baseline and refers to the measurements made by telemetry in the undisturbed mice.

The pairs of readings by the two blood pressure measuring systems obtained in first study yielded weak to almost zero correlation and was not combined with the data obtained in the other studies to assess the correlation coefficient and agreement between the two techniques. The reason for this divergence and the trend observed among the studies will be discussed in this chapter.

Studies number 4 and 5 involved induction of hypertension and the data included the recordings by both techniques before and after the induction of hypertension.

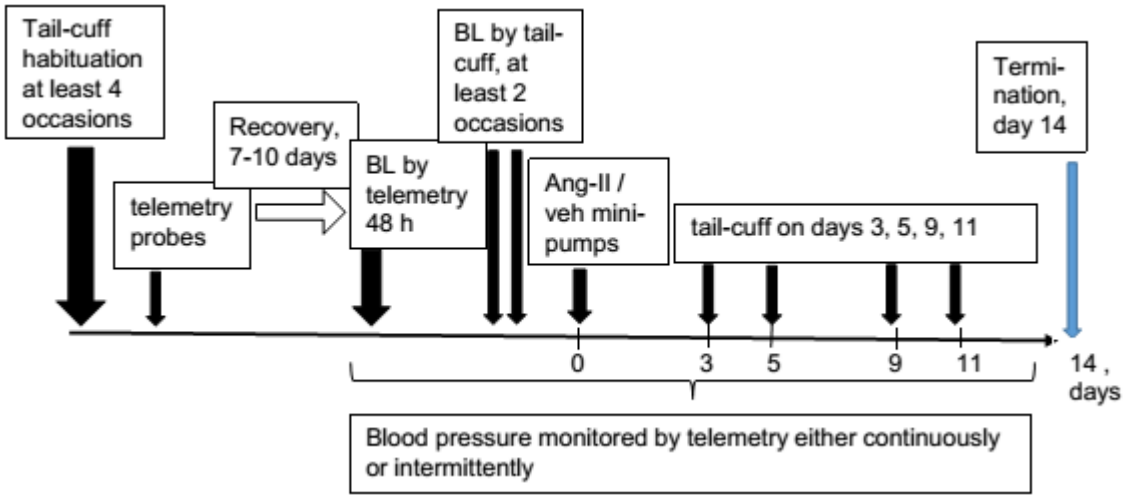


Diagram 5.2. The experimental procedures and their timings used in the hypertension model, as applicable to studies 4 and 5. BL = baseline - refers to the measurements made by telemetry in undisturbed mice, and also to the measurements by the tail-cuff before the implantation of Ang-II or vehicle mini-pumps. *Simultaneous blood pressure recordings by telemetry and the tail-cuff were carried out on at least two occasions before the mice were implanted with the Ang-II or vehicle mini-pumps and thereafter on days 9 and 11 for study number 4, and on days 3, 5, 9 and 11 for the study number 5.*

5.2.3. Simultaneous telemetry and tail-cuff blood pressure recording

Telemetry blood pressure was acquired at the same time as tail-cuff recordings in the same animal by placing the telemetry receiver pad adjacent to the tail-cuff device. Computer clocks on the telemetry and tail-cuff systems were synchronised so that 2 second segments of telemetry recordings were acquired throughout the duration of the recording by tail-cuff. To compare the readings obtained by the two systems, average reading obtained using tail-cuff for each session were calculated. They were compared to the average value calculated for data collected by telemetry during the same period for the same animal. To establish the correlation between the two techniques, individual readings obtained by the two systems were aligned and compared (approximate temporal resolution 2-5 seconds).

5.2.4. Non-simultaneous telemetry and tail-cuff blood pressure recordings

To compare blood pressure readings obtained by telemetry when the mice were neither restrained nor otherwise stressed to those obtained by tail-cuff, we used blood pressure recordings obtained on the same day for each mouse before handling. Typically, 0.5–1-hour period was chosen when the mouse was minimally active 0.5-2 hours before the mouse was handled for the tail-cuff recordings.

5.2.5. Statistical analysis

To assess correlation and agreement between the two techniques to measure blood pressure, the Bland-Altman analysis was used to compare the two techniques (Bland and Altman, 2007), however the difference in the measurements will be compared to the readings obtained by the telemetry, unlike the average between the two techniques as reported by Feng and colleagues Pearson correlation was used to assess linear correlation between the two measuring techniques and regression analysis was used to support the correlation analysis in terms of what proportion of data can be explained by the fitted linear model. A correlation was considered strong for Pearson r> 0.7 (or – 0.7), medium for Pearson r between 0.7 and 0.5 (-0.7 and -0.5), weak for Pearson r< 0.5 and 0.3 (-0.5 and -0.3) and negligible at less than 0.3 (-0.3) (Mukaka, 2012).

GraphPad Prism v5.0 software was used to draw all the graphs in this chapter as well as for all the statistical analysis of the data.

5.3. Results

5.3.1. Simultaneous tail-cuff and telemetry recordings and effect of Ang II

A total of 859 pairs of simultaneous recordings obtained by the telemetry and the tail-cuff techniques with and without Ang II infusion were compared (figures 5.1 and 5.2). The data was fitted to linear model and Pearson correlation analysis was applied. Recordings obtained by the tail-cuff technique were represented as the "response" variable and thus plotted on the y-axis accordingly. Figure 5.1 shows the combined data set that includes both normotensive (at "baseline" or after vehicle infusion) and hypertensive (following Ang II infusion) mice. Pearson correlation coefficients for this combined data set for systolic and diastolic blood pressure was 0.6908 (95% confidence interval 0.6542 to 0.7243) and r=0.6490 (95% confidence interval 0.6085 to 0.6862) respectively. Therefore, the analysis suggests there is medium to strong correlation between the recordings obtained by the two techniques for both systolic and diastolic blood pressure measurements. However, the variation in the recordings obtained by telemetry explains 48% (r^2=0.477) and 42% (r^2=0.42) of variation in recordings obtained by the tail-cuff technique for the peak and trough pressures respectively. The slope for the combined dataset was significantly different from zero and was found to be 0.7629±0.03 for systolic and 0.7796±0.03 for diastolic blood pressure measurements, i.e. 1mmHg change in the recordings obtained by telemetry corresponded to 0.76 – 0.78 mmHg change in the recordings obtained by the tail-cuff. In summary, there is medium to strong correlation between the two systems and just under a half of the variation recorded by one system can be also be reflected by the recordings made by the other system.

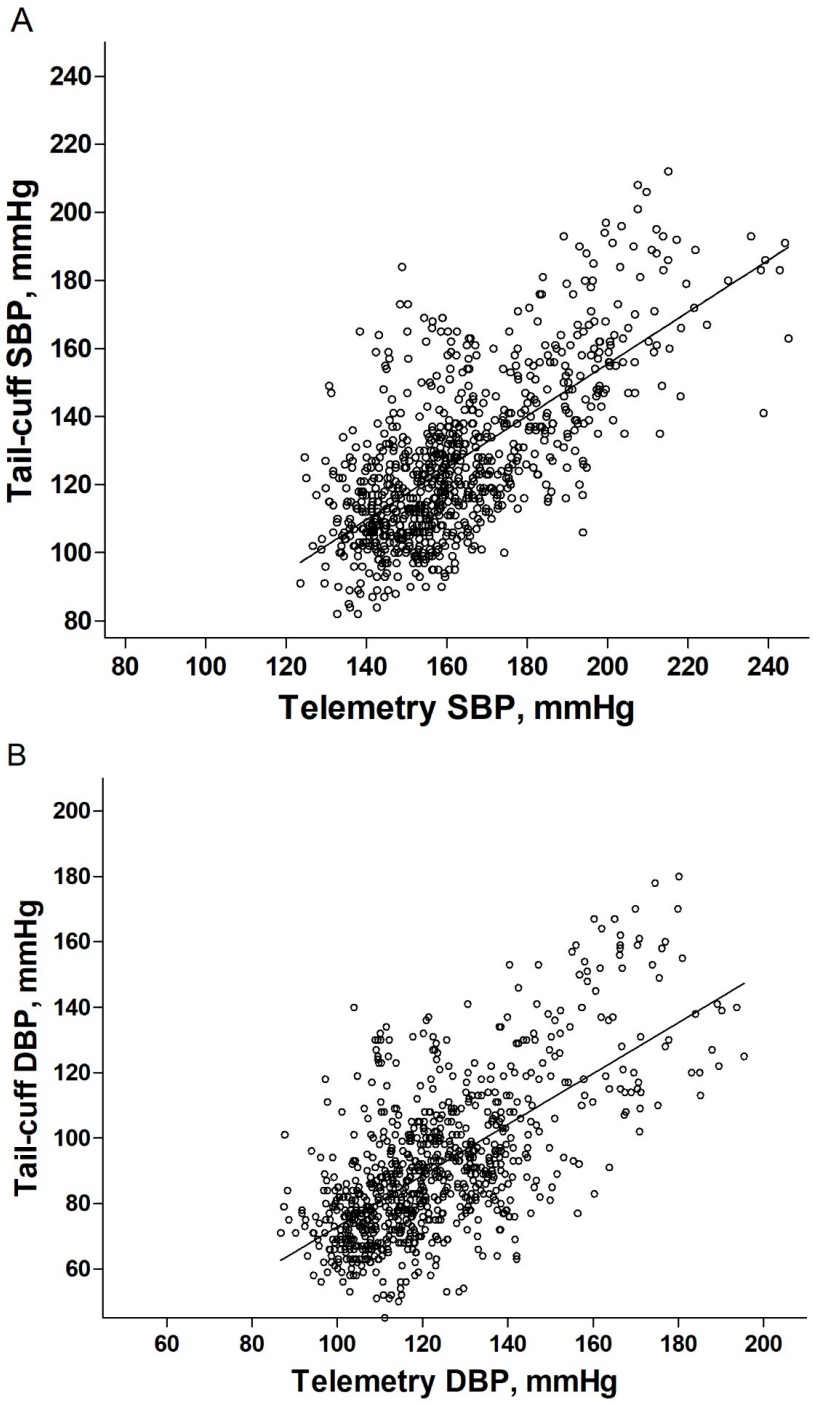


Figure 5.1. Correlation analysis of simultaneous blood pressure recordings made by telemetry and tail-cuff techniques. *Data shows A) systolic (SBP) and B) diastolic (DBP) blood pressure at baseline or following vehicle or Ang II infusion using osmotic mini-pump as part of the studies numbered 2-5 in section 5.2. Each data point represents a pair of time-matched measurement made by the telemetry and the tail-cuff systems in one mouse and the figure shows a total of 859 such paired readings obtained from 21 C57Bl6/J (10 male and 11 female) mice. Solid lines in A) and B) represent the fitted least squares regression line.*

The most immediate and significant observation made when these experiments were started was the difference in the magnitude in blood pressure between the two systems. Using the implanted telemetry, I could see that handling and restraint induced pronounced increase in blood pressure

and heart rate that were maintained during and beyond the tail-cuff protocol was over. The real-time blood pressure recording by telemetry showed that the average blood pressure in "normotensive" mice exceeded 150/115 mmHg, while the simultaneous recording by tail-cuff showed the pressure of 120/80 mmHg. A very similar difference was observed in hypertensive mice: telemetry showed the blood pressure was over 180/140 mmHg, while the simultaneous recordings topped 140/105 mmHg.

Figure 5.2 shows the same data as the figure 5.1 with the exception that the results are differentiated if the mice received Ang II or not (such as before Ang II infusion or following vehicle infusion). Although there was significant overlap between the range of blood pressure values recorded with and without Ang II infusion, on average, higher blood pressure was achieved following Ang II infusion: 144.1±25.5/109.6±26.0 mmHg as recorded by the tail-cuff, and 185.1±19.2/144.1±18.0 mmHg as recorded by telemetry. By comparison, blood pressure in mice who did not receive Ang II infusion was 120.2±17.9/84.9±16.3 mmHg as measured by the tail-cuff and 155.2±13.4/119.4±13.7 mmHg as measured by telemetry.

Weak to moderate correlation was observed for mice who did not receive Ang II (618 data pairs): Pearson r value was 0.4522 (95% confidence interval 0.3871 to 0.5128) for systolic blood pressure and 0.3013 (95% confidence interval 0.2278 to 0.3715) for diastolic blood pressure. Thus only 20% (r^2=0.20) and 9% (r^2=0.09) of the variation in the measurements by the tail-cuff technique was explained by the variation recorded by telemetry for systolic and diastolic blood pressure respectively in "normotensive mice". The correlation coefficient between the two recording systems was stronger following Ang II infusion: Pearson r was 0.7401 (range 0.6771 to 0.79401) for systolic and 0.6806 (95% confidence interval 0.6064 to 0.7431) for diastolic blood pressure. Over a half, or 55% (r^2=0.55) and just under a half, 46% (r^2=0.46) of variation in the systolic and diastolic blood pressure respectively as recorded by the tail-cuff was explained by the variation in recordings by telemetry in hypertensive mice. The slope of the fitted linear regression for systolic blood pressure measurements is 0.60±0.04 for normotensive mice, and 0.98±0.06 for the hypertensive mice. For the diastolic blood pressure measurements, the slope of the linear regression line is 0.43±0.05 for normotensive and 0.98±0.07 for the hypertensive mice. In other words, a change in the measurement by telemetry corresponds to almost identical changes in the measurement by the tail-cuff in hypertensive mice, however the same change in the measurements obtained by telemetry correspond to approximately 60 and 40% change (in the same direction) in the measurements obtained by the tail-cuff for systolic and diastolic pressures respectively in normotensive mice.

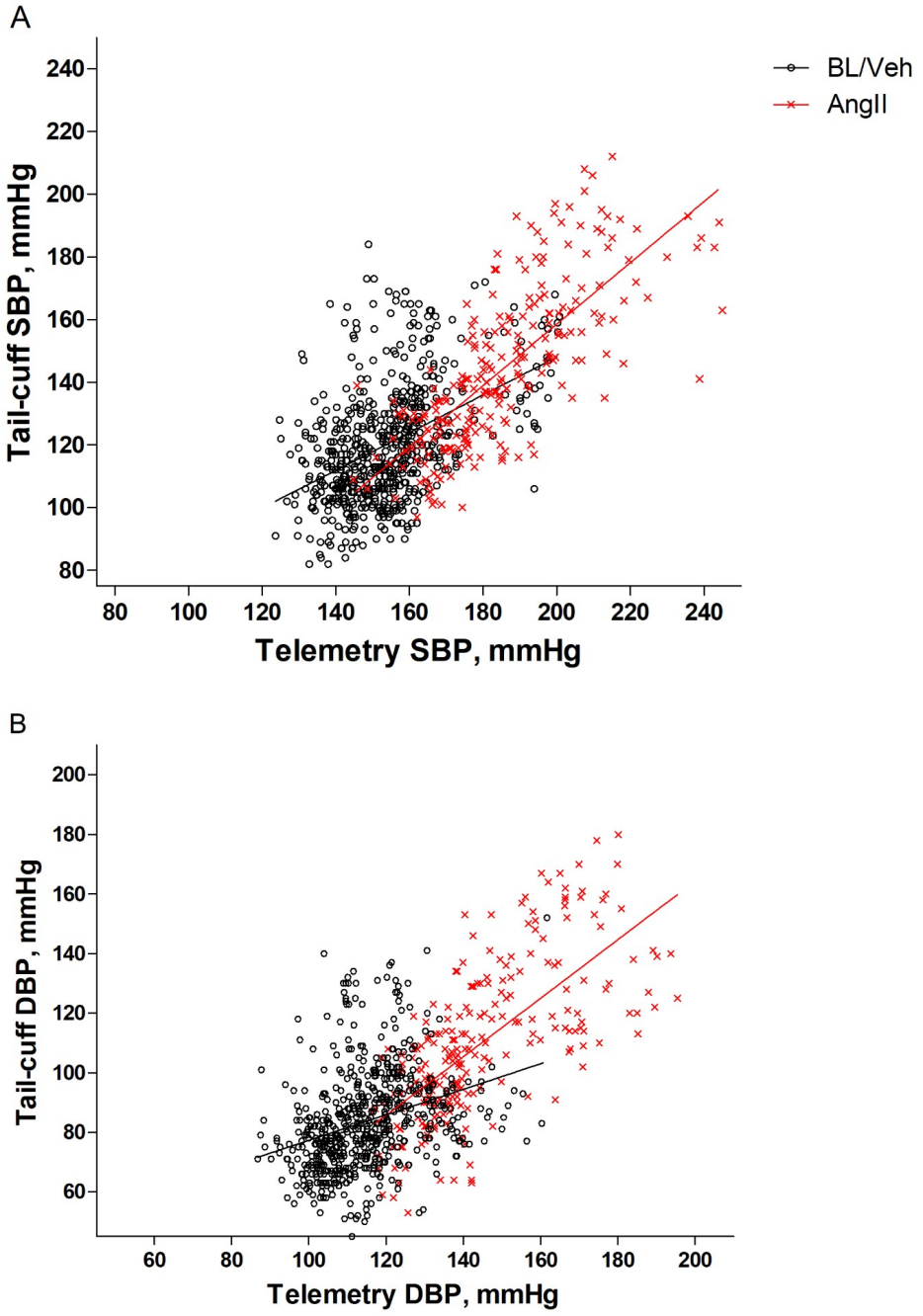


Figure 5.2. Correlation analysis of simultaneous blood pressure recordings made by telemetry and tail-cuff techniques. *Data shows A) systolic (SBP) and B) diastolic (DBP) blood pressure at baseline or following vehicle infusion (BL/Veh, clear circles), and following Ang II infusion using osmotic mini-pump (red crosses). Each data point represents one of the 859 time-matched measurements made by telemetry and the tail-cuff systems: 618 BL/Veh (clear circles) and 241 points following Ang-II infusion (red crosses) obtained from 21 C57Bl6/J (10 male and 11 female) mice. Solid lines in A) and B) represent the fitted least squares regression lines for BL/Veh dataset (black line) and the data obtained in mice following Ang-II infusion (red).*

Table 5.2. Summary of the correlation analysis of telemetry and tail-cuff recordings made in normotensive and hypertensive mice.

	SBP		DBP	
	BL/Veh	AngII	BL/Veh	AngII
Number of XY Pairs	618	241	617	241
Pearson r	0.4522	0.7401	0.3013	0.6806
95% confidence interval	0.3871 to 0.5128	0.6771 to 0.7924	0.2278 to 0.3715	0.6064 to 0.7431
P value (two-tailed)	P<0.0001	P<0.0001	P<0.0001	P<0.0001
R squared	0.2044	0.5478	0.09081	0.4632
Slope	0.6024 ± 0.04788	0.9794 ± 0.05756	0.4298 ± 0.05484	0.9820 ± 0.06837
95% Confidence Intervals	0.5086 to 0.6963	0.8666 to 1.092	0.3223 to 0.5373	0.8479 to 1.116

It is apparent that the data obtained in normotensive mice has weaker correlation and the changes in the blood pressure recorded by the tail-cuff are explained to a lesser degree by the changes recorded by the telemetry. I scrutinised this difference and subdivided the data based on the chronological order.

Table 5.3. Comparison of studies performed during this project.

A. Systolic Blood Pressure

Chronological study number	*1*	*2*	*3*	*4*	*5*
Number of XY pairs	*467*	386	45	173	253
Pearson r	*0.1553*	0.3752	0.4809	0.7431	0.7801
95% Confidence Interval	*0.0654 to 0.2426*	0.2863 to 0.4577	0.2181 to 0.6787	0.6680 to 0.8033	0.7267 to 0.8242
R squared	*0.02411*	0.1408	0.2313	0.5522	0.6086
Number of mice	*3 (males)*	4 (males)	5 (females)	8 (4 males/ 4 females)	4 (2 males/ 2 females)
Slope	0.2160 ± 0.063	0.6697 ± 0.084	1.115 ± 0.309	0.8033 ± 0.055	0.8695 ± 0.044
95% Confidence intervals	0.0911 to 0.3409	0.5046 to 0.8347	0.4893 to 1.740	0.6949 to 0.9117	0.7833 to 0.9558
Mean difference between telemetry and the tail-cuff recordings	36.75±19.4	30.12±18.2	42.50±11.1	39.38±17.3	40.08±16.4

B. Diastolic Blood Pressure

Chronological study number	*1*	*2*	*3*	*4*	*5*
Number of XY pairs	*467*	*386*	*45*	*173*	*253*
Pearson r	*0.2496*	*0.2771*	*0.2877*	*0.5983*	*0.7293*
95% Confidence Interval	*0.1626 to 0.3329*	*0.1823 to 0.3667*	*-0.006463 to 0.5360*	*0.4934 to 0.6860*	*0.6659 to 0.7823*
R squared	*0.06232*	*0.07676*	*0.08276*	*0.3579*	*0.5319*
Number of mice	*3 (males)*	*4 (males)*	*5 (females)*	*8 (4 males/ 4 females)*	*4 (2 males/ 2 females)*
Slope	*0.2875 ± 0.051*	*0.3261 ± 0.057*	*0.7416 ± 0.376*	*1.118 ± 0.114*	*0.8520 ± 0.050*
95% Confidence intervals	*0.1861 to 0.3889*	*0.2130 to 0.4392*	*-0.0181 to 1.501*	*0.8940 to 1.341*	*0.7531 to 0.9509*
Mean difference between telemetry and the tail-cuff recordings	*39.15±17.0*	*31.38±17.8*	*36.33±11.6*	*27.74±21.5*	*34.94±17.1*

There is a distinctive trend for the correlation to become stronger as the project was progressing (the first study took place in early 2015 and the last study ended before the middle of 2016). The last two studies were similar in terms of the correlation coefficient and other parameters. Therefore, I went on to separate the data by mouse gender (and hypertensive status) in the last two studies.

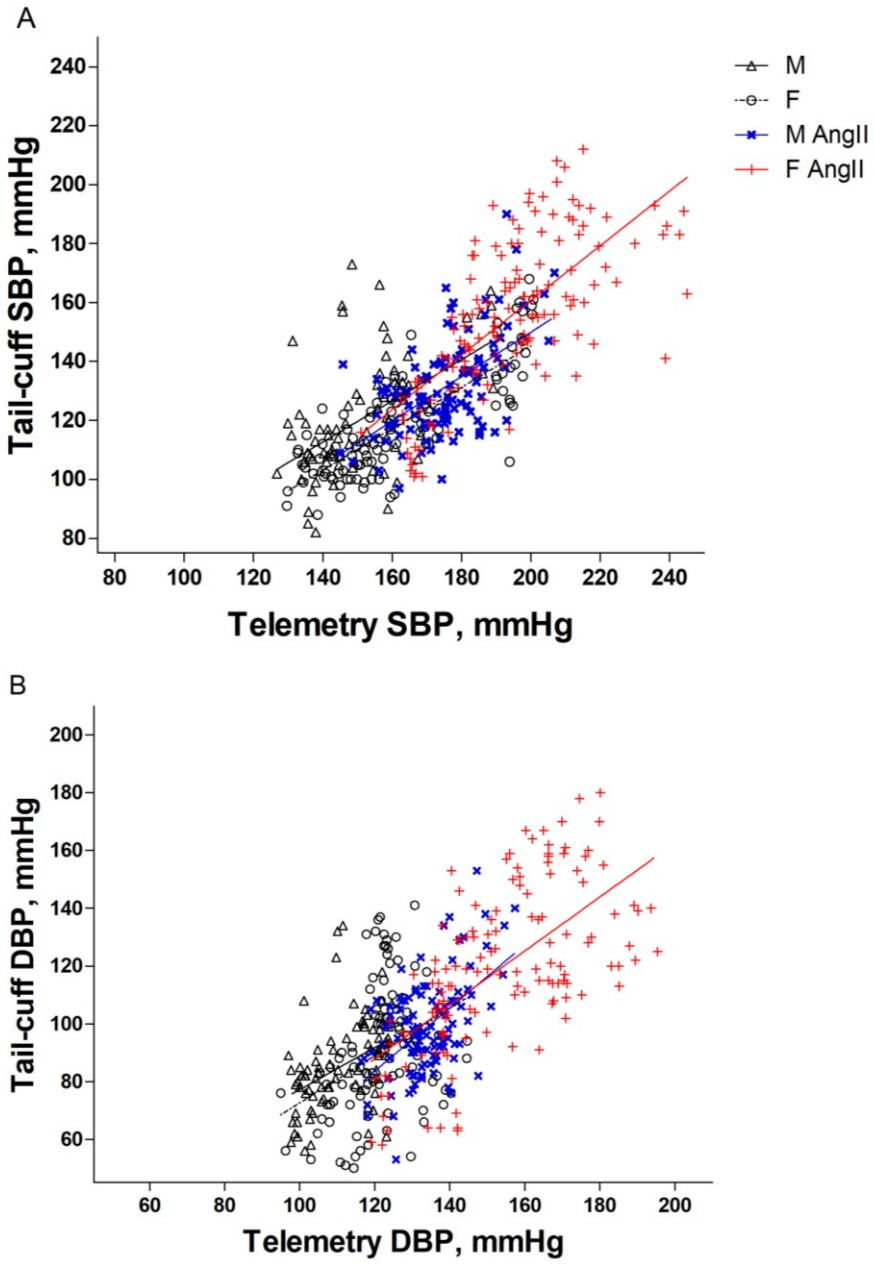


Figure 5.3. Comparing correlation of simultaneous blood pressure recordings made by telemetry and tail-cuff techniques between male and female normotensive and hypertensive mice obtained in studies numbered as 4 and 5. *Data shows A) systolic (SBP) and B) diastolic (DBP) blood pressure at baseline or vehicle infusion (BL/Veh, Male [M] – clear triangles, females [F] -clear circles), and following Ang II infusion using osmotic minipump (F - red crosses, M – dark blue rotated crosses). Each data point represents one of the 426 paired measurements by telemetry and the tail-cuff in 12 C57Bl6/J (6 male and 6 female) mice.*

Table 5.4. Summary table of the correlation analysis of telemetry and tail-cuff recordings made in normotensive and hypertensive male and female mice (studies 4 and 5 as numbered in table 5.2).

	Males		Females	
SBP	Baseline / vehicle	Ang-II	Baseline / vehicle	Ang-II
Number of XY Pairs	83	109	102	132
Pearson r	0.5199	0.5544	0.8090	0.6939
95% confidence interval	0.3426 to 0.6615	0.4089 to 0.6724	0.7292 to 0.8671	0.5934 to 0.7732
R squared	0.2703	0.3074	0.6545	0.4816
Slope	0.6994 ± 0.1277	1.092 ± 0.1690	0.7022 ± 0.05102	0.9266 ± 0.08432
Slope 95% Confidence Intervals	0.4449 to 0.9539	0.5361 to 0.9698	0.6008 to 0.8036	0.7613 to 1.092

	Males		Females	
DBP	Baseline / vehicle	Ang-II	Baseline / vehicle	Ang-II
Number of XY Pairs	86	109	100	132
Pearson r	0.3883	0.5298	0.3303	0.6240
95% confidence interval	0.1922 to 0.5546	0.3794 to 0.6528	0.1431 to 0.4947	0.5071 to 0.7183
R squared	0.1508	0.2806	0.1091	0.3893
Slope	0.7271 ± 0.1883	1.092 ± 0.1690	0.8169 ± 0.2358	0.9344 ± 0.1026
Slope 95% Confidence Intervals	0.3521 to 1.102	0.7567 to 1.428	0.3483 to 1.285	0.7333 to 1.136

Comparing the results for male and female mice before and after Ang II infusion (using only the data from the last two studies), stronger correlation and overall results concordance following Ang II infusion it is not confirmed. The first three studies yielded weaker correlation and these studies did not have the simultaneous recordings following Ang II infusion available. Therefore, it is concluded that it is the contribution of those first three studies with weaker correlation that affected the data for the normotensive mice. There is no apparent trend for the difference between the telemetry and tail-cuff recordings to change among the five studies, so overall validity of the recordings obtained in the first three studies is thought to be robust, however the reason for this variation will be discussed later.

5.3.2. Testing agreement between telemetry and the tail-cuff methods

As the next step to understand the relationship between the recordings obtained by the two techniques, I analysed how the agreement, or the difference between telemetry and the tail-cuff changes compared to the central blood pressure measured by telemetry. I decided to separate the data from the last two studies and the other studies for this analysis.

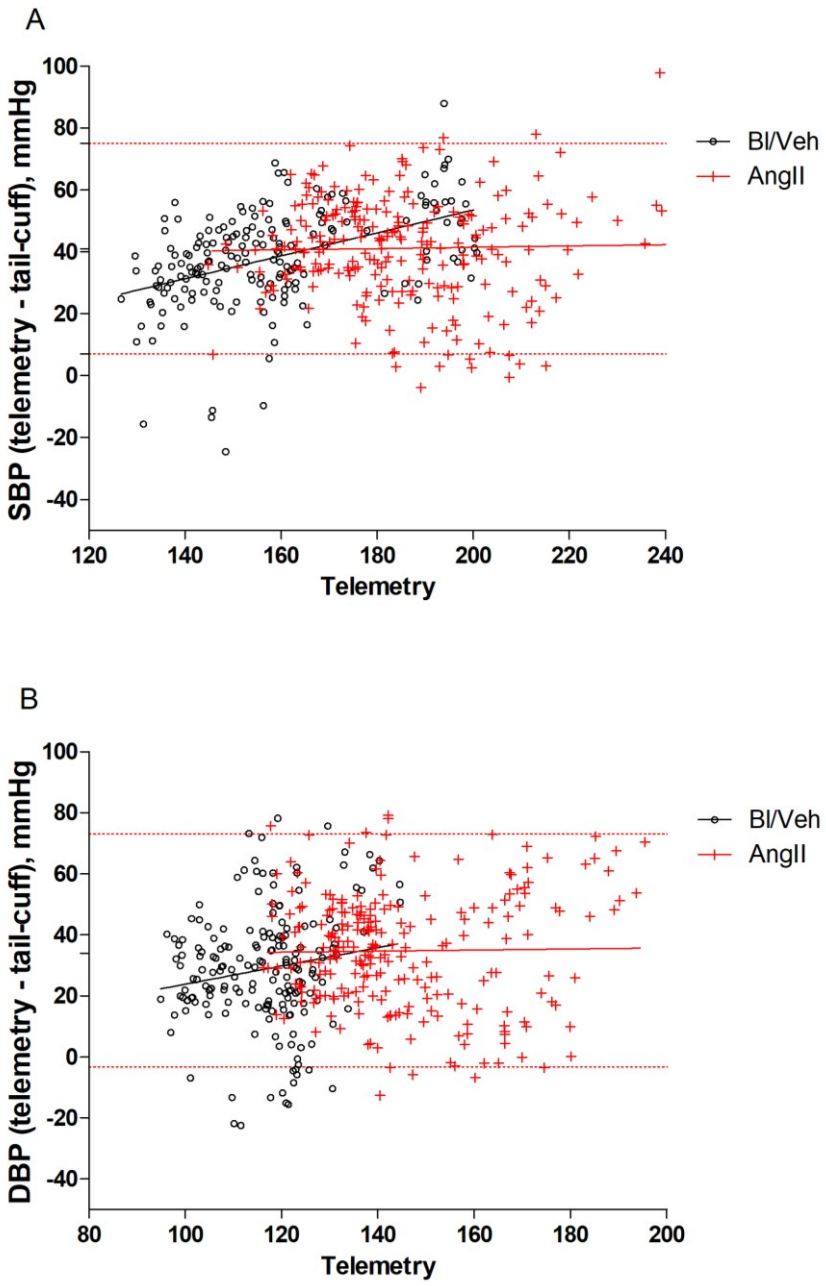


Figure 5.4. Bland-Altman plot of simultaneous systolic (A), diastolic (B) blood pressure recordings obtained during the 4th and 5th studies. *Systolic blood pressure (SBP) or diastolic blood pressure (DBP), as measured simultaneously by the telemetry and tail-cuff at baseline (clear circles) and following Ang II infusion (red crosses). Each data point represents one of the 426 paired measurements by telemetry and the tail-cuff in 12 C57Bl6/J (6 male and 6 female) mice. Solid black lines in figures A and B represents the fitted regression line for the measurements made for "normotensive" mice and solid red line represent the fitted regression line taken for the measurements made in hypertensive mice. Dashed red lines represent ±2 standard deviations from the mean difference.*

Figure 5.4 represents the data obtained as part of the 4th and 5th studies in this project and includes the measurements made in male and female "normotensive" and hypertensive mice. Despite some potential artefacts, telemetry is the most reliable and accurate way to measure central blood pressure available today, while the tail-cuff technique estimates central blood pressure from the more peripheral tail artery. Therefore, I decided to plot the difference between

the methods versus the respective measurements made by telemetry, as the reference gold-standard method.

Difference between the telemetry and tail-cuff measurements made in "normotensive" mice (before Ang II or following vehicle infusion), according to the fitted regression line, appears to increase as the measurements made by telemetry increase. The slope of the fitted regression line for systolic blood pressure is 0.3702±0.058, r^2=0.1965, and 0.2964±0.14, r^2=0.0259 for diastolic blood pressure. Moreover, the data for systolic and diastolic blood pressure did not pass the normality test (D'Agostino & Pearson omnibus normality test). By comparison, the slope of the fitted linear regression for the measurements obtained in hypertensive mice was not significantly different from zero for both systolic and diastolic pressure measurements (slope>0.03, r^2>0.001) and the data passed the normality test. Therefore the 95% confidence (±2 standard deviations) intervals are plotted only for the measurements obtained in the hypertensive mice.

A Bland-Altman plot of the data confirms the significant bias already highlighted earlier in this chapter, i.e. the tail-cuff method tends to underestimate the systolic and diastolic blood pressure by approximately 38mmHg and 29mmHg respectively. The lower and upper limits where the 95% of data are contained are 5.2 and 70.4 mmHg differences for the systolic blood pressure and -10.5 and 67.7 mmHg for the diastolic blood pressure.

Figure 5.5 shows the difference between telemetry and the tail-cuff (also plotted against the measurements made by telemetry) for studies 1-3 that were obtained in normotensive mice only. The average difference is very similar across all the studies and averages at around 35 mmHg for both systolic and diastolic pressures. The difference also appears to increase at higher central blood pressure values as recorded by telemetry, as also seen in the figure 5.4 for the "normotensive" mice. The difference between the techniques is uniform for the hypertensive mice (figure 5.4).

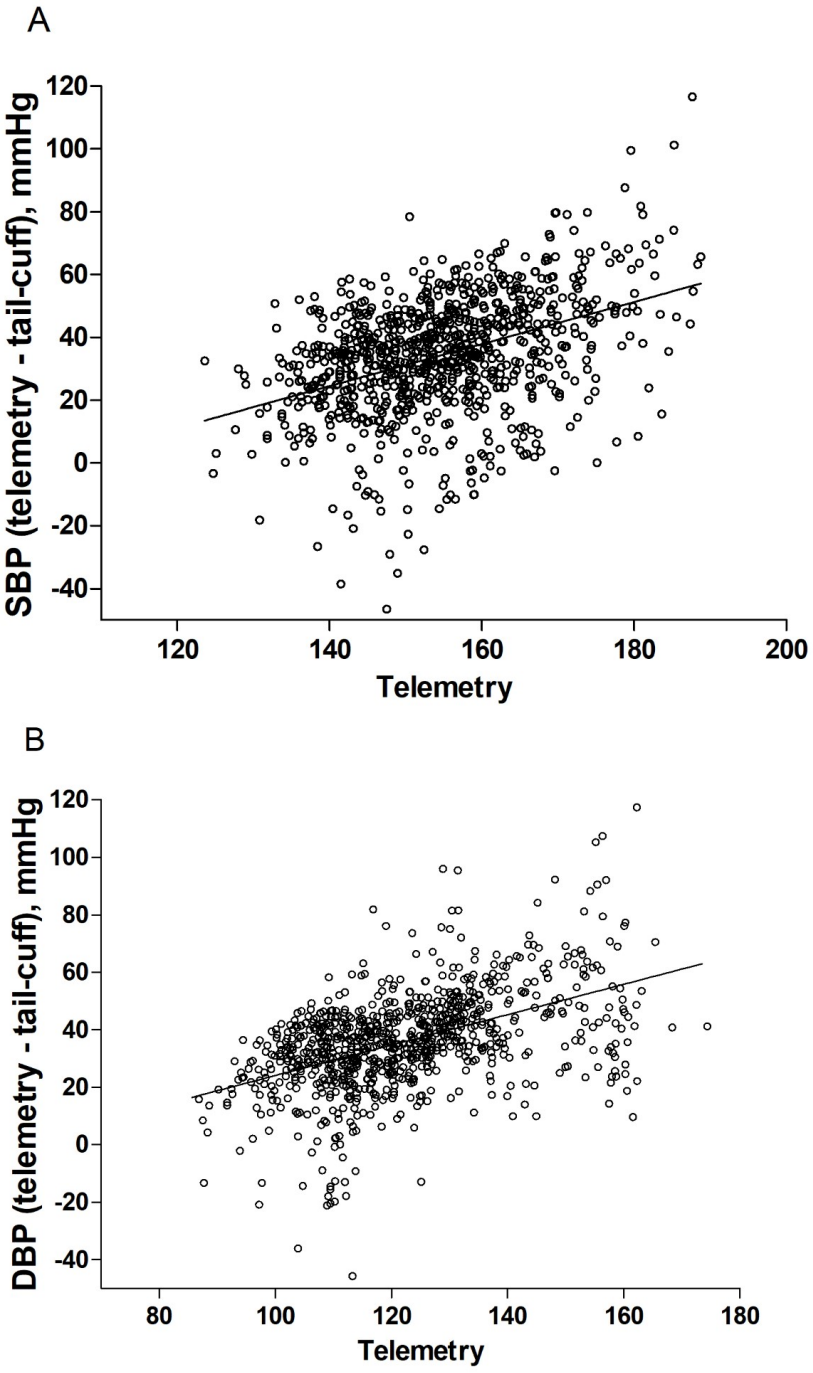


Figure 5.5. Bland-Altman plot of simultaneous systolic (A), diastolic (B) blood pressure recordings obtained during the 1st, 2nd and 3rd studies. *Systolic blood pressure (SBP) or diastolic blood pressure (DBP), as measured simultaneously by the telemetry and tail-cuff in normotensive mice. Each data point represents one of the 898 paired measurements by telemetry and the tail-cuff in 12 C57Bl6/J (7 male and 5 female) mice. Solid black lines in figures A and B represents the fitted regression linear line for these measurements.*

5.3.3. Analysis of non-simultaneous recording obtained by telemetry and tail-cuff techniques

To compare the recordings acquired non-simultaneously by both techniques, I calculated the average for the average values for each recording session for each mouse as recorded by the tail-cuff and as recorded by telemetry before the mouse was disturbed or handled for the tail-cuff

technique on the same period that the tail-cuff readings took place. Each data point in figure 5.6 below represents recordings matched by the day of recording for each mouse.

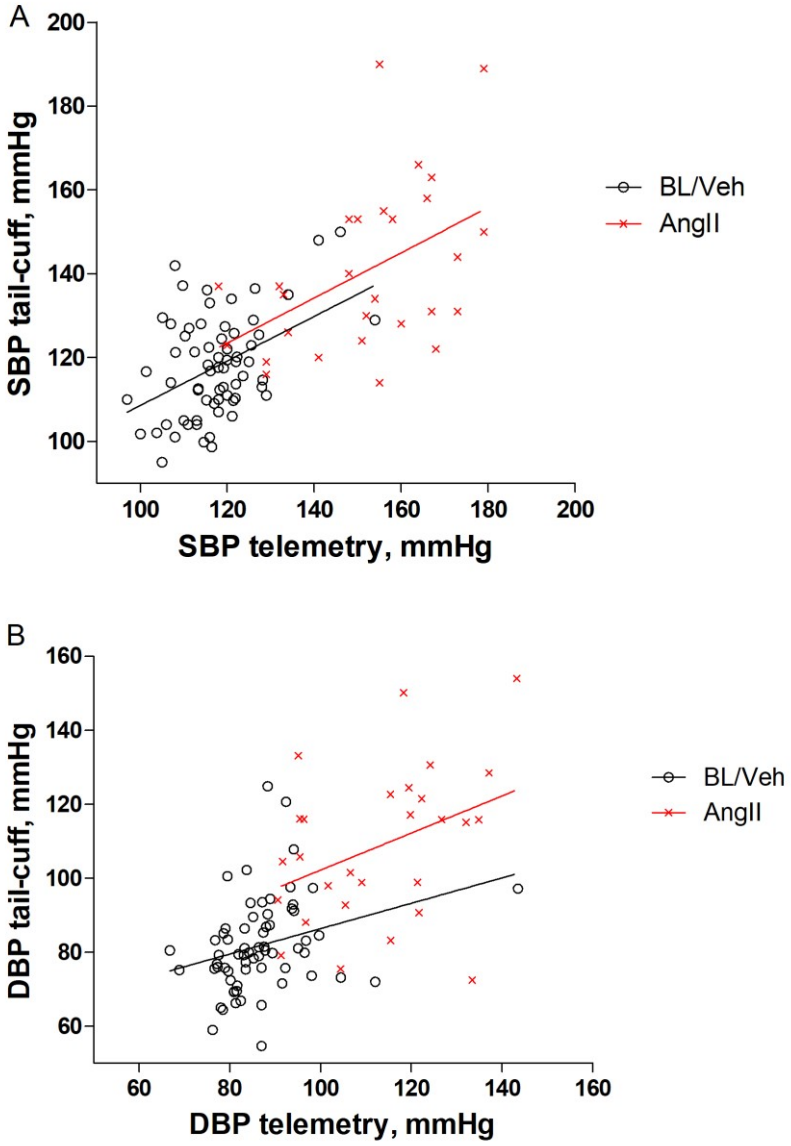


Figure 5.6. Comparison of recordings acquired non-simultaneously by telemetry and tail-cuff techniques. *Data shows A) systolic (SBP) and B) diastolic (DBP) blood pressure at baseline or following vehicle (clear circles) or Ang II (red crosses) infusion using osmotic mini-pump. Each data point represents one of the 94 calculated mean values for each day of recording obtained for 21 C57Bl6/J (10 male and 11 female) mice. Black solid line represents fitted regression line during "baseline" or vehicle infusion ("normotensive" mice) and the solid red line represents fitted regression line for the data points obtained following Ang II infusion ("hypertensive" mice).*

Table 5.5. Summary table of the correlation analysis of non-simultaneous telemetry and tail-cuff recordings made in normotensive and hypertensive male and female mice.

Non-simultaneous recordings	SBP		DBP	
	Baseline/Vehicle	Ang II	Baseline/Vehicle	Ang II
Number of XY Pairs	66	28	66	28
Pearson r	0.4345	0.4623	0.2860	0.3777
95% confidence interval	0.2151 to 0.6122	0.1078 to 0.7126	0.0471 to 0.4939	0.0052 to 0.6581
R squared	0.1888	0.2138	0.08179	0.1426
Slope	0.5316 ± 0.1377	0.5397 ± 0.2030	0.3429 ± 0.1436	0.5011 ± 0.2409
95% Confidence Intervals	0.2563 to 0.8068	0.1223 to 0.9570	0.05586 to 0.6299	0.0057 to 0.9964

Correlation analysis reveals that there is weak to medium correlation between the tail-cuff and telemetry recordings made on the same day. Only up to 20% (r^2=0.19 and 0.21 for normotensive and hypertensive mice respectively) of variation in systolic measurements made by the tail-cuff can be explained by the corresponding variation in the recordings made by telemetry. And only 8% and 14% for normotensive and hypertensive mice respectively of variation in diastolic pressure measurements made by one technique correspond to similar changes observed with the other technique.

5.3.4. Analysis of agreement for non-simultaneous recordings made by telemetry and the tail-cuff

There is on average smaller differences between the measurements made by the two techniques on the same day, but not at the same time. Thus, the mean difference for systolic readings is close to zero for systolic (-0.6±11.4 mmHg) and diastolic (0.8±14.9 mmHg) pressure readings made in in “normotensive” mice and it is 11.4±19.5 and 4.3±20.8 mmHg for systolic and diastolic pressure measurements made in “hypertensive” mice (where the tail-cuff tends to underestimate the central pressure measurements). Standard deviation in these recordings is not too dissimilar to the one observed for the simultaneous measurements. Figure 5.7 shows the data presented in the form of the Bland Altman plot. The difference does not appear to be uniformly distributed, which violates the main assumption for the Bland Altman analysis. Therefore, no such analysis was formally carried out. The bias between the techniques is based on the average difference between the techniques and the confidence intervals are calculated as ±2 standard deviations from the calculated mean value.

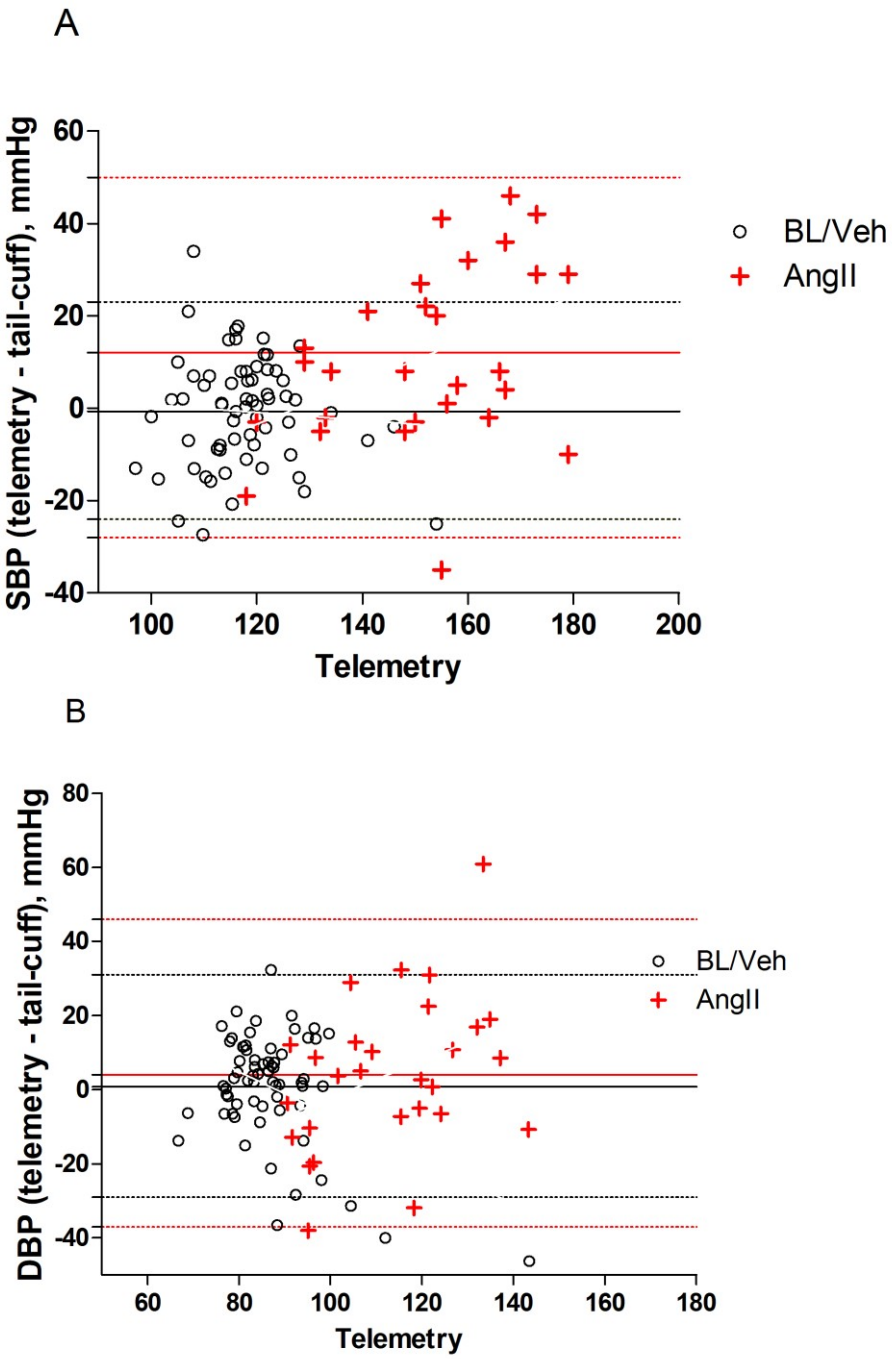


Figure 5.7. Bland-Altman plot of non-simultaneous systolic (A), diastolic (B) blood pressure recordings by telemetry and the tail-cuff. *Systolic blood pressure (SBP) or diastolic blood pressure (DBP), as measured on the same day, but not at the same time, by the telemetry and tail-cuff at baseline (clear circles) and following Ang II infusion (red crosses). Each data point represents one of the 94 calculated mean values for each day of recording obtained for 21 C57Bl6/J (10 male and 11 female) mice. Solid black and red lines in figures A and B represent the calculated average difference between measurements made by the two techniques for "normotensive" and hypertensive mice respectively. Dashed black and red lines represent ±2 standard deviations from the mean difference.*

5.3.5. Analysis of the simultaneous and non-simultaneous recordings for blood pressure measurements in normotensive and hypertensive mice in a typical Ang II-induced hypertension protocol

To further explore how both techniques compare in terms of the way the data would be typically presented in a hypertension study, I calculated the average value for each recording session based on the accepted readings as recorded by the tail-cuff and the average for the period of the tail-cuff session as recorded by telemetry. The results are shown in figure 5.8 and show the data for 7 male C57Bl/6 mice obtained over a period of approximately 2 weeks.

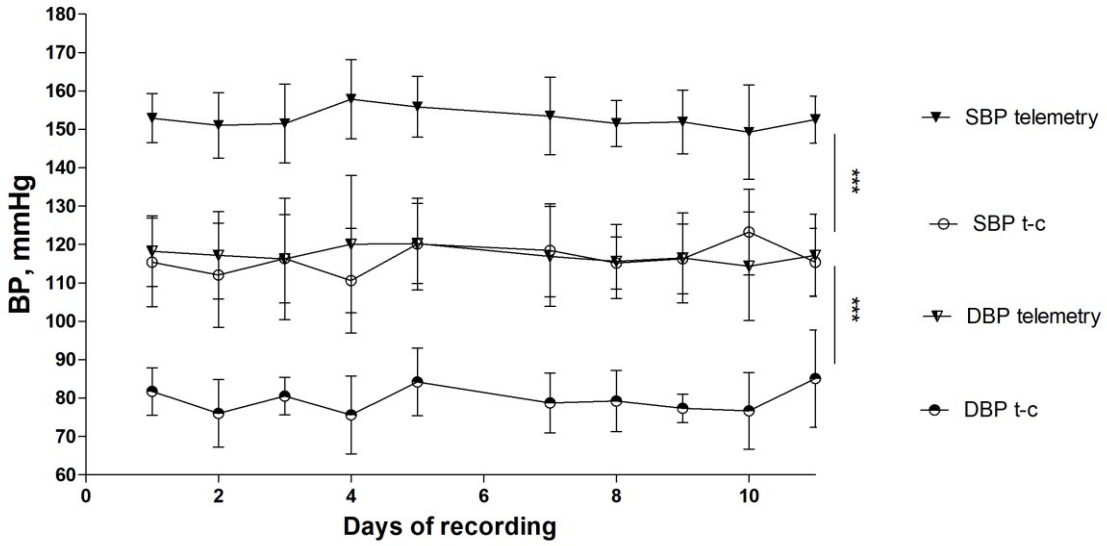


*Figure 5.8. Comparison of recordings acquired simultaneously by the two techniques in normotensive mice (male C57Bl6/J, n=7). Each data point represents an average value for the readings acquired during a recording session on each day when these recordings were made for each mouse, and for 7 mice in total. RM-ANOVA analysis revealed there is significant difference between the measurements made by the two techniques (***p<0.001) for both systolic and diastolic pressure.*

Interestingly, the systolic blood pressure as recorded by the tail-cuff is remarkably similar to the diastolic blood pressure as recoded by telemetry during the same period. One can see that the difference of approximately 30-40 mmHg is maintained on all the days of recording. Fairly stable readings of between 113±13 - 123±11 mmHg for systolic blood pressure were obtained by the tail-cuff and 149±9 - 156±10 mmHg for systolic blood pressure as obtained by telemetry. Diastolic blood pressure ranged between 78±9 - 85±7 mmHg and 114±14 -120±16 mmHg for the tail-cuff and telemetry respectively. Although the measurements obtained by the two techniques do not closely follow some of the day-to-day variation, the difference between the two techniques remains relatively similar.

I went on to compare the non-simultaneous measurements made by the two techniques. I calculated an average value for each session as recorded by the tail-cuff (as already shown in

figure 5.8) and the average blood pressure values as recorded by telemetry during an undisturbed period before the tail-cuff protocol took place on the same day (figure 5.9). The two techniques show fairly similar results for blood pressure measurements. Although the difference between the two techniques can reach some 20 mmHg average difference for this set of results, the large data spread makes this difference not significant.

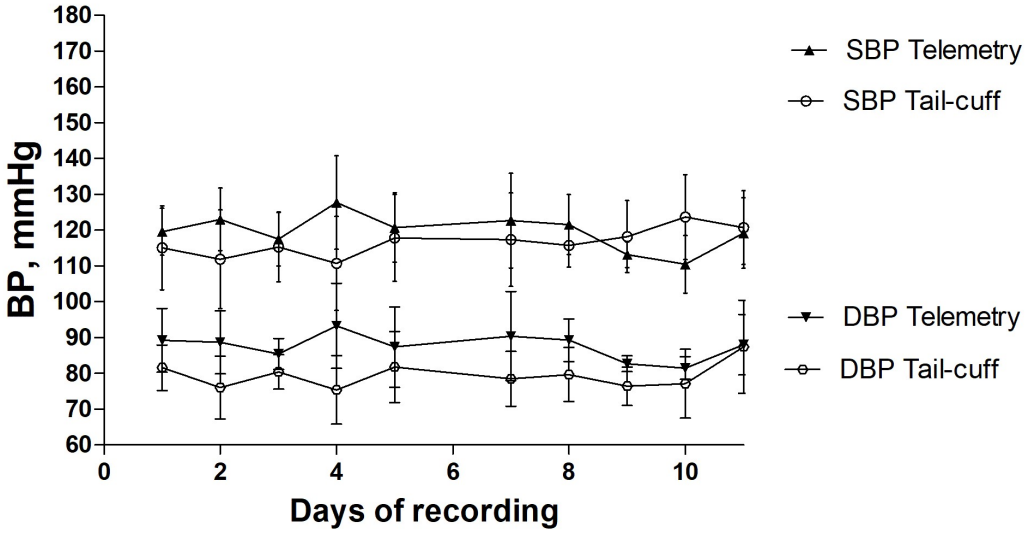


Figure 5.9. Comparison of recordings acquired non-simultaneously by the two techniques in normotensive mice. *SBP and DBP measured by telemetry (filled triangles) before the animals were disturbed; SBP and DBP measured by the tail-cuff (open circles). Each data point represent mean ± SD for 7 male C57Bl6/J normotensive mice. RM-ANOVA revealed no significant difference between the measurements made by telemetry or the tail-cuff.*

The most important question in evaluating the tail-cuff technique to measure blood pressure in hypertension projects is whether this technique can reliably establish the onset of hypertension. Figure 5.10 shows the blood pressure measurements obtained by the tail-cuff and telemetry, both simultaneously and non-simultaneously in a typical Ang II-induced hypertension protocol.

Ang-II infusion caused approximately 30 mmHg increase in both systolic and diastolic blood pressure as can be seen in the measurements made by telemetry before the mice were disturbed for the tail-cuff protocol (non-simultaneous recordings by telemetry) and the measurements made by telemetry during the tail-cuff protocol. The tail-cuff technique, according to this data, reflects the same changes in blood pressure as observed by telemetry.

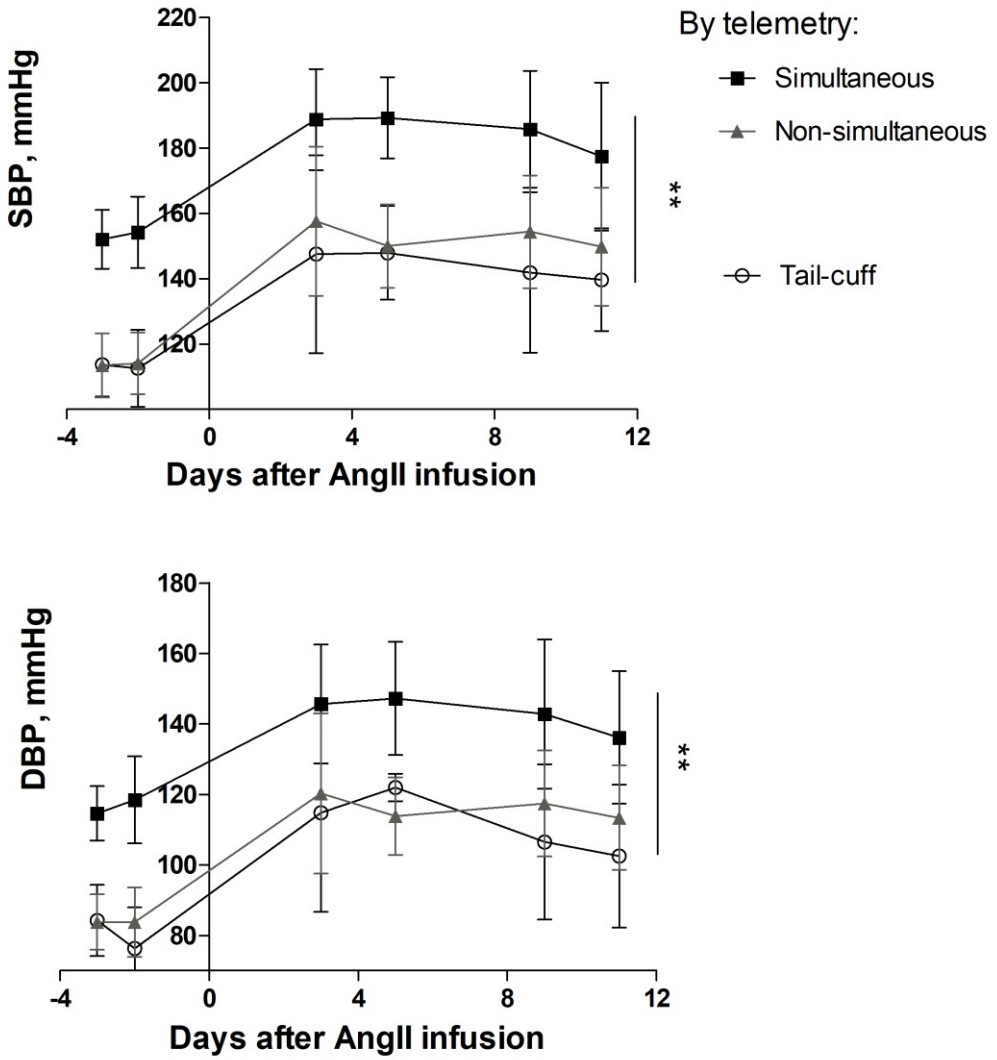


Figure 5.10 Comparison of recordings acquired simultaneously and non-simultaneously by the two techniques at baseline and in the Ang II hypertension model. *A) SBP measured by telemetry before the animals were disturbed (grey bar) and during tail-cuff protocol (filled bar), compared to the SBP readings obtained by the tail-cuff technique (clear bar) in the same mouse without Ang II infusion (n=6); ***p<0.001 telemetry recordings during the tail-cuff protocol vs tail-cuff recordings and telemetry undisturbed. B) SBP and C, DBP determined by the two techniques before and following Ang II infusion: measurements by telemetry before the animals were disturbed (grey triangles), during the tail-cuff protocol (filled squares) and measurements by the tail-cuff technique (clear circles). Values are mean ± SD for 8 male and female C57Bl6 mice on days -3, -2, 9, 11; and 4 mice on days 3 and 5; **p<0.01 vs telemetry recordings during the tail-cuff protocol (RM-ANOVA).*

These results suggest that, despite the large difference between the simultaneous measurements made by telemetry and the tail-cuff technique, the tail-cuff technique is able detect hypertension, at least in Ang II – induced model, and the tail-cuff is also able to estimate blood pressure in "normotensive" mice that is otherwise comparable to the central blood pressure measurements made by telemetry.

5.4. Discussion

The main objective of this chapter was to compare and correlate the measurements made by telemetry and the tail-cuff techniques. These measurements were made at the same time by the two techniques and referred to as "simultaneous" recordings; measurements made not at the same time, but on the same day, were also compared and correlated. The rationale behind including this data was as follows. Telemetry allows recording of blood pressure in freely moving undisturbed mice and the research projects where telemetry is used, record blood pressure in undisturbed mice. It was thought to be important to include the data I had available in the undisturbed mice to compare both with the recordings obtained using the tail-cuff technique, and with the recordings made by the telemetry, when the mice are disturbed for the tail-cuff technique. It is also important to bear in mind that the two techniques record the blood pressure from the two different vascular beds: directly from central aortic arch by telemetry and indirectly from the more peripheral tail artery by the tail-cuff. It may be hypothesised that the stress-induced pressure changes are not reflected in the same way in the two vascular beds. Therefore, I thought it was important to compare the tail-cuff measurements both to the simultaneous and non-simultaneous recordings made by telemetry.

Simultaneous measurements by the two techniques differ considerably in magnitude, between 30 – 40 mmHg on average. This is a significant change in the measured parameter that in effect typically renders all mice "hypertensive" during the tail-cuff protocol. This stress-induced change makes this blood pressure increase comparable to blood pressure that may be observed in mice following Ang II infusion when the mice are undisturbed. Ang II infusion typically further increased the blood pressure during the tail-cuff protocol. The difference in the simultaneous measurements between the tail-cuff and telemetry remains fairly constant throughout and in the end the tail-cuff is able to detect hypertension, albeit at a constantly lower value than the corresponding simultaneous measurements made by telemetry. Simultaneous recordings made by the two techniques show medium to strong correlation for both systolic and diastolic pressures, at least as evident from the last two studies in this project. It is not entirely clear why only a weak correlation was achieved in the earlier study, especially in the very first study. There is no other apparent difference in the recording between the earlier and the later studies. It may be due to the synchronisation error between the telemetry and the tail-cuff computers. The other reason may be optimisation of the heating protocol that had to be adapted to be used for the simultaneous telemetry and the tail-cuff recordings. Since the standard heating platform could not be used in conjunction with telemetry, several adaptations had to be made. Since the tail-cuff protocol heavily relies on adequate heating, it is a likely source of error in the tail-cuff

readings. It is interesting though that there is progressive trend for stronger correlation as the project progressed and it points me back to the figure 3.4B in Chapter 3, which highlights the importance of operator training to be able to obtain sufficient number of valid readings by the tail-cuff.

Certain electrical equipment interfered with the telemetry recordings and this caused gaps in telemetry data. Although precautions were taken to exclude this electrical interference, in some cases, interference occurred, and this caused artefactual measurements by telemetry. These readings were relatively easy to identify and exclude. Artefactual readings typically included negative values for a measured parameter and/or abnormally high values for pressure wave (above 300 mmHg). These artefactual readings were excluded and are not believed to have affected the data interpretation.

The non-simultaneous recordings obtained with the two systems have weaker correlation. This is perhaps not surprising, as the recordings were made at different timepoints, such that the "non-simultaneous" telemetry readings were taken before the mice were disturbed for the tail-cuff. Although it was aimed to include the periods when the mice were at rest, it was not always possible and the telemetry recordings in this data set may have been affected by random and transient factors that would not be reflected in the subsequent recordings. The average difference between the non-simultaneous tail-cuff and telemetry recordings is markedly smaller, although still with quite large variation in this difference overall.

To my knowledge, no direct measurements from mouse tail artery have been made. It is known that the blood pressure in the rat tail artery are some 17 mmHg lower than in the central artery (Wang et al., 2013). In silico modelling and some experimental data in mice (Aslanidou et al., 2016) suggests that the blood pressure also gradually decreases from centre to the periphery in the mouse, however the magnitude of this change is not clear. It may be that this difference in the simultaneous recordings made by the telemetry and the tail-cuff is due to this inherent pressure difference in the two different vascular beds that was coincidentally assumed to be reflective of the normal physiological pressure otherwise expected to be seen in the central circulation. I cannot exclude that this difference may be due to how the tail-cuff was calibrated. I could not devise a direct method how to check this hypothesis, however I performed a calibration check of the tail-cuff system against a mercury manometer, which suggested that the pressure reading by the tail-cuff system had no such calibration error.

Chapter 6.

General Discussion

6.1. Summary of major findings

The development of blood pressure telemetry for mice at the turn of the 21st century has opened up new possibilities in hypertension research. However, the technique has limitations, as detailed in the Introduction (chp 1). The tail-cuff technique, on the other hand, is technically simpler and cheaper but the animals need to be restrained. These factors are understood to cause stress and otherwise affect the normal haemodynamics, which understandably caused concerns over how reliable the measurements obtained by the tail-cuff are (Mills et al., 2000) and are the central area of study in this thesis.

Although the implantable telemetry system offers a range of refinement features for blood pressure recording, the tail-cuff remains a valuable alternative and, as such, is recommended for certain applications in cardiovascular research by the American Heart Association (Kurtz et al., 2005b). With some initial falling out of favour with the advent of telemetry, the tail-cuff technique becomes more widely recognised for the advantages that is does have and its appropriate use is still advocated in present day publications (Drüeke and Devuyst, 2019; Luther and Fogo, 2019). Although figure 6.1 shows the results of searching only one database, I believe the results of this search reflect a wider valid trend. The number of publications that report the use of telemetry rapidly overtook the number of publications that relied on the tail-cuff technique in the first decade telemetry was developed for mice and by 70% in the second decade. However, the use of the tail-cuff technique continues to rise.

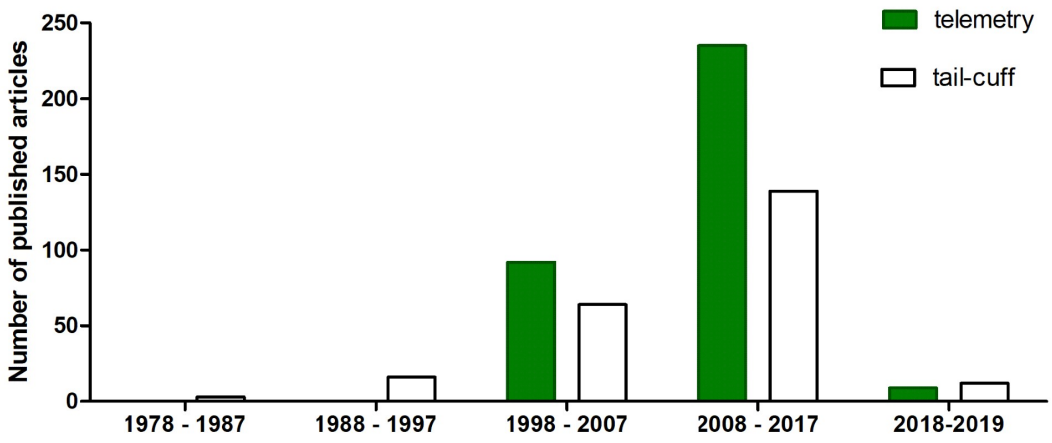


Figure 6.1. The number of articles that reported the use of the telemetry or the tail-cuff technique. *The PubMed database was searched using the terms "telemetry mouse blood pressure" and "tail-cuff mouse blood pressure" for the time periods shown.*

In summary, I found that the handling techniques and sex of the investigator has little effect on the measurement on mouse blood pressure using the tail cuff. However, the simultaneous measurement of blood pressure by tail cuff and telemetry reveals a remarkable divergence between the two techniques. This related to the restraint as well as other steps of the technique. We do not believe this has been adequately reported previously. The data generated in this quantitative study supports the American Heart Association recommendations for blood pressure measurements in mice; (Kurtz et al., 2005a) that the tail-cuff technique can detect changes in blood pressure, similar to those observed in the Ang II-induced hypertension model here. They recommend that it is used for screening of large numbers of animals and long-term studies where the use of the more accurate telemetry techniques have limitations, as discussed above. We believe that researchers should be aware of our findings when designing their studies that involve the tail-cuff technique.

The major findings for the tail cuff are summarized in the table below.

Table 6.1. Summary of key findings and recommendations for the tail-cuff protocol

Chapter	Intervention	Influence on central BP and/or HR or other measured parameters
3	Three handling methods investigated	All handling techniques induced large, yet very similar increases in BP and HR during handling and the following steps of the tail-cuff protocol as compared to the reference baseline period. No apparent reduction in cardiovascular responses was observed following repeated handling by any of the techniques.
3	Mice were handled by male and female researchers in the context of the tail-cuff protocol	Male and female handlers caused very similar increases in BP and HR during handling and thereafter. Although smaller increases in HR were observed in response to non-handling interventions by the handler familiar to the mice, these differences were obliterated once the handling took place.
4	Other interventions associated with the tail-cuff protocol (presence of the researcher in the room, moving cage, restraint with and without heating, and tail-cuff inflations) as well as handling, were compared in their effect on central BP, HR and core body temperature	All interventions, apart from the mere presence of the researcher in the room, induced appreciable increases in BP, HR and core body temperature. Restraint appeared to cause the largest and sustained increase in all the measured parameters. Warming the mice to 33-36°C or tail-cuff inflations did not cause further increase in BP or HR.
4	Central BP and HR were repeatedly recorded in male and female C57Bl6/J mice during the tail-cuff protocol	Male and female mice showed similar increases in central BP and HR during the tail-cuff protocol. No apparent reduction in the measured parameters was observed following at least 15 exposures to the tail-cuff protocol in the male mice.
5	Simultaneous and non-simultaneous measurements of blood pressure were performed by telemetry and the tail-cuff in normotensive and hypertensive male and female mice.	Large differences (approximately 40 mmHg) between the measurements made at the same time in the same mouse by the tail-cuff and telemetry were observed for both systolic and diastolic blood pressure. There was medium to strong correlation between the measurements obtained by the two techniques for simultaneous recordings. Correlation was weak – to medium for the first studies number 1-3 and became progressively stronger by study number 5. Measurements obtained by the tail-cuff system were similar to those obtained by telemetry on the same day in undisturbed mice Both telemetry and tail-cuff systems were able to detect the progression of hypertension in mice following chronic Ang-II infusion.

6.1.1. Summary of recommendations for the tail-cuff technique based on the findings

In spite of the large difference between the simultaneous measurements obtained by telemetry and the tail-cuff in the same mouse, the tail-cuff system is able to estimate blood pressure in normotensive mice and reflect the progression of hypertension following Ang-II infusion.

Handling and restraining the mice for the tail-cuff invariably induces large increases in blood pressure and heart rate. There is no clear advantage of any of the tested handling techniques in terms of the impact of handling and the other interventions associated with the tail-cuff on the cardiovascular responses. Maintaining optimal ambient temperature (around 33-35°C) is also important to improve reliability of the measurements performed by the tail-cuff technique as estimates of central blood pressure. Although there is no evidence that the mice habituate to the tail-cuff technique in terms of the magnitude of responses to the protocol, it is important that the mice are familiar with the handler and the handler is familiar to the tail-cuff technique.

6.2. Handling and the influence of stress

The goal of this project was to refine the tail-cuff technique. Since it was not possible to eliminate the restraint, one of the main lines of enquiry was to improve the way the mice are handled during the tail-cuff protocol, and potentially understand what other refinements to the protocol can be introduced to alleviate the stress that the tail-cuff technique is understood to cause to the mouse.

Stress is a broad term that is can be defined as the "biological response to perceived threat to homeostasis" (Moberg and Mench, 2000). Stressful situations cause fear and anxiety, that are accompanied by profound physiological changes that affect most, if not all organs and systems, including the cardiovascular system, which is at the centre of this project (Johnson et al., 1992).

Hurst and West (2010), as discussed throughout the thesis, showed that handling the mice using the tunnel reduces anxiety compared to mice handled by the tail, supported by (Gouveia and Hurst, 2017). The handling techniques tested in the first experiment were based on those of Hurst and West (2010). Because the tail-handling was not the method I normally used, I replaced it with the "tailcup" method. This handling method also involved initially picking up the mouse by the tail, however the mouse was almost immediately placed into the palm of the hand in the technique that I used.

I relied on the blood pressure recordings by the tail-cuff alone as stress markers at the first stage of the project. The results (figure 3.4) showed that the handling techniques tested had very similar effect on blood pressure, irrespective of gender.

Considering the multitude of variables involved in handling the mouse, I defined the elements of handling that may have different (or stress) liability that a particular handling technique may cause. I identified an element of restraint that the "tail" handling, as described by Hurst and West

(2010), involved. The way the mouse is caught: by the tail or encouraged to walk into the tube (or the "tunnel"), or into the hand, as shown in the supplementary videos to the publication (Hurst and West, 2010) may be another element important for stress liability. It is also important that a particular handling technique can be relatively easy to use in all mice.

With reproducibility and inclusion of factors that may bear different stress liability in mind, I then compared the "tunnel", or tube handling with the "tail" handling. The results of this experiment in male mice (as shown in figure 3.5) again showed no difference in blood pressure or faecal pellets deposited while the mice were in restraint and their blood pressure was recorded by the tail-cuff.

Since I started using the tail-cuff technique to record blood pressure in mice, I was puzzled by the fact that the restrained mice appeared stressed during the recording sessions, however their blood pressure fluctuated typically around the 110-120mmHg mark for the systolic and 70-85 mmHg for the diastolic pressures. There was no apparent trend for the blood pressure, as measured by the tail-cuff, to decrease from the supposedly hypertensive stressed state during the first recording occasions.

These questions raised my doubt in the validity of the measurements that the tail-cuff technique could produce. Moreover, there seems to be a lack of information in the literature to directly compare the tail-cuff with a direct technique to measure blood pressure in the conscious mouse, with only one relatively recent study (Feng et al, 2008) that at least in part directly compared the two techniques. This led to planning the blood pressure telemetry experiments.

All handling techniques induced similarly large increases in blood pressure and heart rate as measured by telemetry during the handling period and thereafter. These results were contrary to my hypothesis, which was that the handling technique by the tail that induced higher anxiety in the mouse (as demonstrated by Hurst and West, 2010) would also induce larger increase in blood pressure and heart rate during the handling itself and then during the rest of the tail-cuff protocol.

There are some important differences in the stress markers and the experimental design between the studies by Hurst and West (2010) and these experiments. Firstly, the experiments by Hurst and West measured anxiety and the main end-point in these experiments was blood pressure and heart rate. Secondly, I used a cross-over design such that the mice were habituated to handling and experienced all handling techniques compared. The cross-over design offers greater sensitivity to detect a change in the same subject, thus reducing the effect of between-subject

variability. It also requires fewer animals, which is important from the ethical point of view and simplifies availability and cost considerations of the mice especially with regards to telemetry experiments. I believe the cross-over design was still justified in these experiments.

I believe the design of the handling experiments was adequate to allow the conclusion that the handling techniques tested in these experiments ("tail", "tail-cup" and "tunnel") had similar acute effects on blood pressure and heart rate at the time of handling and later on during the rest of the tail-cuff protocol duration. These effects were similar over the five days that each handling technique was tested for (i.e. none of the techniques offered a more favourable habituation profile).

The rationale behind distinguishing the intervention stages identified within the tail-cuff protocol was that each stage entailed a component that may be aversive to the mouse. Handling is undoubtedly a major intervention that hugely impacts on the cardiovascular system. Placing the mouse into the restraint tube may introduce an additional element to capture, where the mouse is "encouraged" into the tube, i.e. it is made to do something that it does not intend to do. Researchers do normally aim to find the best way to encourage the mouse into the restraint. These results suggest that being able to place the mouse into the restraint on first attempt does prevent further increase in blood pressure, however it does not have a further impact on the haemodynamic response once in the restraint.

Interestingly, it was my observation, as well as feedback from other researchers who routinely handle the animals, that the tube handling method did not always appear to be the most optimal way of handling. The same home cage enrichment tube appeared aversive to some mice when used for handling and involved several attempts to lift the mouse in the tube.

I examined the data obtained in the handling experiments at 10-sec time resolution in order to see any trends within the periods of the tail-cuff protocol and I also included the recordings from the point when the mouse cage lid lifted. This revealed that all the measured parameters had noticeably increased by the time the cage lid was lifted, and the period between the lid removal and handling saw the largest increase in heart rate, to approximately 150 bpm. Handling caused a further rise in this parameter that apparently reached its maximum before the mice were placed into the restraint. The blood pressure changes were more gradual, and each step caused a further increase. The blood pressure peaked following several minutes into the restraint period. Both blood pressure and heart rate tended to decrease towards the end of the 15minute restraint period I used in these studies, however they did not appear to fall below the level attained at the beginning of the restraint period for blood pressure or the period just before handling for the

heart rate. I believe this relative attenuation of the responses indicates that perhaps the mice give up struggling, however I do not believe that the mice "acclimatised" to the restraint within the 15-minute recording period, nor that longer restraint period was required to achieve this. Other studies that demonstrated that the stress response (as measured by the level of stress hormones) only becomes greater if the restraint period is prolonged (Sorge et al., 2014) in mice. A much longer period of restraint used in rats revealed high level of stress hormones after 2 hours in restraint (Kvetnanský and Sabban, 1998).

6.3. Environmental influences, including operators

Sorge et al (Sorge et al., 2014) showed that exposure to male odours induced a significant increase in stress hormone corticosterone levels, whereas the response to female odour was indistinguishable from control. Our experiment with the male and female researchers handling the mice for the tail-cuff protocol (on different days) showed that human males and females evoked very similar changes in blood pressure and heart rate during the handling and later on during restraint and recording by the tail-cuff (figure 3.17).

In the same study, Sorge et al (2014) also point out that the effect of the male odours was diminished by the concomitant presence of female odours. Because I, the female researcher was present in the room at least at some stages of the experiment, this may have affected the results. Furthermore, Sorge et al also pointed out that the researcher's gender only mattered in observational studies, i.e. where no handling as such in involved, and did not affect the results when substances were administered via injection (i.e. handling, restraint and injection took place). I believe that this also means that the researcher's gender should not have any significant effect on physiological responses in the context of the tail-cuff protocol, as supported by the cardiovascular data generated in these experiments.

Another outcome of the recording sessions that involved myself (a familiar researcher) and other researchers who were not familiar to the mice, was an observation that mice developed smaller increases in heart rate when a familiar researcher approached, however this difference disappeared by the time the animals were handled. This also reinforces the previous conclusion that researcher's gender and whether the researcher is familiar to mice, does not influence the haemodynamic response to handling in the mouse.

I had the opportunity to measure the effect of these interventions on core body temperature, an important tightly regulated physiological parameter that may also be used as a stress marker (Cabanac and Briese, 1992). I decided to further evaluate the impact of the interventions that

did not involve the restraint (human presence, moving the cage, handling) and the relative contribution of heating and tail-cuff occlusions to the restraint. I proceeded using the tail-cup or tube handling at the next stage of the project.

It was the familiar researcher (myself) that entered the room in this set of experiments. "Moving the cage" intervention in this series of experiments had similar effect on blood pressure and heart rate to removing the cage lid as shown in the previous set of experiments: within the 1st minute of the event the heart rate was remarkably similar to that induced by restraint, however the effect on blood pressure and core body temperature were more moderate. Handling induced an even greater rise in blood pressure and heart rate, yet marginally lower than that achieved during restraint. All interventions that involved restraint induced the largest increases in blood pressure and heart rate that were maintained throughout the 15-minute restraint period. These were very similar throughout regardless if the heating was used and if tail-cuff inflations were added after the 5th minute in restraint.

Mouse core body temperature was affected in a similar manner to blood pressure and heart rate. It was increased when the cage was moved and more so when handling and restraint took place. In my hands, the restraint with warming and the tail-cuff technique were found to induce an increase in core body temperature. However, surprisingly, when no warming was used, the initial rise in the core body temperature was attenuated, possibly due to the cooling effect of the non-heated room temperature tube. Although the tube's temperature was similar to the mouse's ambient temperature, contact with the tube material could interfere with the temperature homeostasis of the mouse and exert the apparent cooling effect and require a longer period in the restraint to acclimatise. Therefore, pre-heating the tubes to 33-36°C is recommended to avoid perturbations in core temperature.

It is essential to warm mice to record blood pressure using the tail-cuff method, as recommended by the equipment manufacturer (Daugherty et al., 2009). This is achieved by using an inbuilt heating platform that also holds the restraint tubes. It is assumed that warming the animals to 33-36°C allows higher blood flow to the tail. The thermo-sensitive nature of the tail means that it is otherwise constricted at the usual ambient temperature of the laboratories and animal holding rooms (Williams et al., 2002; Swoap et al., 2004; Daugherty et al., 2009), not allowing the tail-cuff recordings to take place. This is not surprising as ambient temperatures of the animal holding and procedure rooms have previously been shown to be below the thermoneutral range for mice, which is suggested to be approximately 30°C. (Swoap et al., 2004) At thermo-neutrality, mice have lower blood pressure and heart rate (Swoap et al., 2004) and vagal tone driven control of the heart rate. (Swoap et al., 2008) Warming using a heat box to 27°C has been suggested to be

a stressing factor, causing increased blood pressure and heart rate. (Swali et al., 2010). The results suggest that heating mice up to 36°C is not a stressing factor per se, as the unheated restrained mice show a similar blood pressure and heart rate.

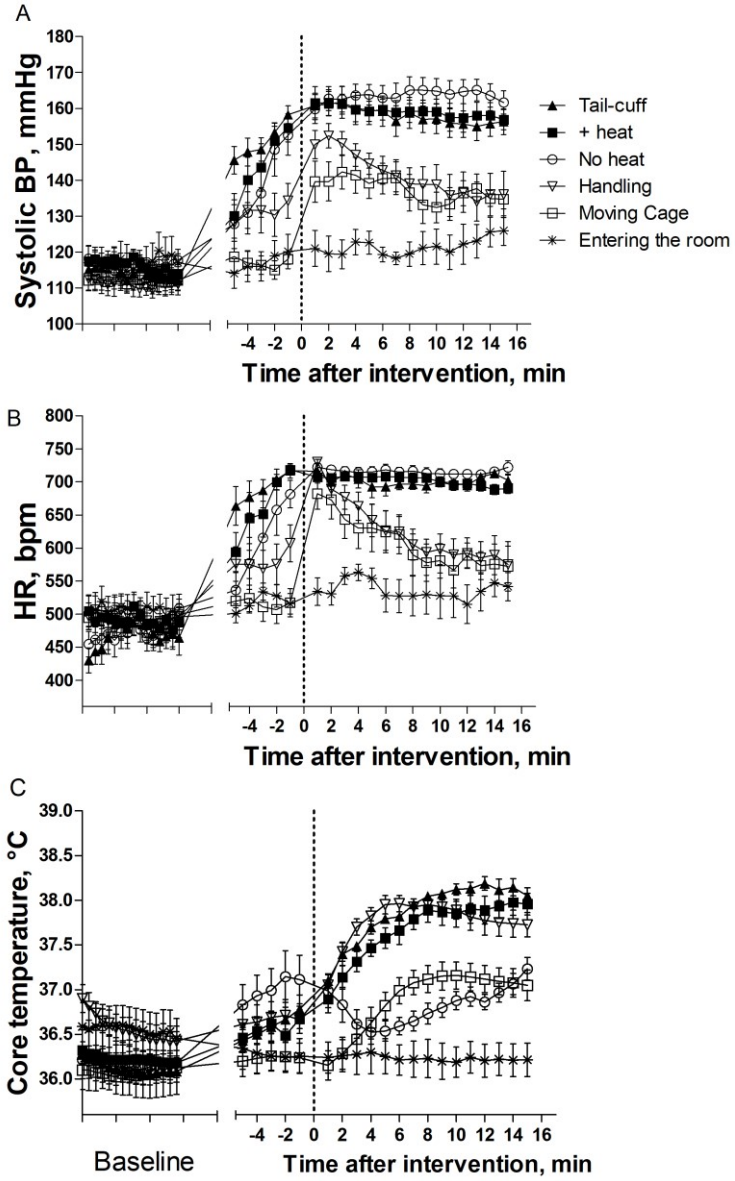


Figure 6.2. Summary of the cardiovascular and core body temperature changes in response to interventions associated with the tail-cuff technique as determined by telemetry (adapted from figures 4.1 -4.4 in this thesis). *A) Changes in systolic blood pressure (SBP). B) Heart rate (HR). C, Core [body] temperature. Interventions took place within the following periods: presence of the researcher in the room from between 0-3min, cage was moved at 0 min, handling took place between 0-1 min, restraint with and without heating and tail-cuff started at 0 and lasted until 15 min. Values are mean ± SEM for 4 animals for each intervention.*

Interestingly, the core body temperature following handling was indistinguishable to that induced by restraint with heating / tail-cuff. This may be due to the nature and reactivity of the markers used (i.e. temperature and cardiovascular) in response to the different stressors. The increase in

core temperature observed in this study in the female mice was similar to the one observed in the male mice in other studies (Cabanac and Briese, 1992; Keeney et al., 2001a).

Considering the summary of the cardiovascular telemetry data (figure 6.2), restraint is most stressful to the mouse and has the largest impact on blood pressure. It is worth noting however, that handling lasted between less than 30 seconds to 1 minute in these experiments. Handling mice over 2.5 minutes was not tested in this project. When the mice were handled for up to 2.5 minutes, the blood pressure and heart rate were maintained at a higher level during this period, and I assume should the mice be handled for longer, the cardiovascular measurements will be maintained similar to restraint. Therefore, it may be that handling and restraint may differ in duration and not in the causative factor that elicits the stress response. Cabanac and Briese used rectal probes to measure core temperature and they report average temperature of 38.4°C on the first day, 15 minutes after the first handling occasion. The same response was attenuated by approximate 1°C on the 17th day of handling. Although I did not test for habituation to handling alone in the experiments that measured temperature, the repeated restraint for the tail-cuff technique appeared to bring on lower increases in core body temperature that may indicate there is some habituation to restraint, however not as measured using the cardiovascular markers.

Switching on telemetry probes may also affect both cardiovascular system and core body temperature. This appears to be caused by the fact that the researcher has to approach the cage and shift the cage to bring the magnet in proximity to the mouse. The effect appears to be driven by activity, with no habituation, as the disturbance to the mouse may be random. The observed effect however may last up to half an hour and irrespective of gender.

My results also indicate that male and female mice have similar haemodynamic responses at all the stages of the tail-cuff protocol. Although I did not study core body temperature changes during the tail-cuff protocol and its various components in male mice, male mice appear to react similarly to handling (Cabanac and Briese, 1992; Keeney et al., 2001b). Although Chen and Yu (Chen and Yu, 2018) demonstrate that male mice develop higher hyperthermia in response to heat exposure, the heating platform and that of the restraint tubes was maintained between 33-36°C and not 39.5°C as used by Chen and Yu; therefore I anticipate that male mice have similar temperature changes in response to the tail-cuff protocol. At baseline, however, I observed that female mice have higher heart rate both during the day and night than the male mice, while blood pressure remained nearly identical. Other studies that used both males and females report that males have higher blood pressure, yet lower heart rate in C57Bl6/J mice (Barsha et al., 2016) between 3 and 5 months of age. Another study that investigated the cardiovascular circadian

rhythms in only male mice reported similar blood pressure to the one I observed in our colony of mice, yet they report higher heart rate that is more comparable to the one I observed in the females in the same strain of mice (Mousa et al., 2014). It is generally accepted that there are important gender differences in blood pressure regulation (Wang et al., 2017; Huxley and Kemp, 2018; Reckelhoff, 2018; Beale et al., 2019; Reckelhoff et al., 2019) including adaptation to exercise (Bassareo and Crisafulli, 2019), however both genders appear to have similar responses to the tail-cuff and some other stressors.

It is standard to train mice so that they become accustomed to the handling and restraint (Daugherty et al., 2009). The interventions starting from the cage lid removal caused significant perturbations to blood pressure and heart rate compared to the resting state. It appears that restraint, as it was at least used in the context of the tail-cuff protocol, had the greatest impact and overrides any potential benefit of a less aversive handling technique or researcher's gender. We observed approximately 30-45% increase in blood pressure and 50 to 75% increase in heart rate that was maintained throughout the restraint period. Other researchers show similar changes in mice (Gross and Luft, 2003) and rats (Bidani et al., 1993; Xu et al., 1996), and also show that there is no reduction in blood pressure or heart rate as a result of repeated exposure to the technique (Gross and Luft, 2003; Batchu et al., 2015). On the contrary, repeated restraint and tail-cuff recording over 5 consecutive days has been used by others as a method to induce stress (Batchu et al., 2015). However, it is our observation that mice become familiar with both the handling and the researcher, and this may facilitate certain experimental procedures.

Although increases in blood pressure, heart rate and body temperature are accepted markers of stress. intensive activity, such as during the dark hours, is also associated with higher blood pressure, heart rate (Li et al., 1999; Agarwal, 2010; Sheward et al., 2010; Mousa et al., 2014; Barsha et al., 2016) and core body temperature in both male and female mice (Tokizawa et al., 2015). These experiments did not distinguish the noxious stress from other types of stress.

6.4. Improvement in knowledge, compared with related research

These findings, for the first time, to my knowledge, directly compare the two techniques and are the first to show that there is a highly significant difference between simultaneous tail-cuff and central blood pressure telemetry readings.

The VPR tail-cuff system used in this study has been previously validated against telemetry in the only one study by Feng et al, (Feng et al., 2008) with the conclusion that there was negligible difference between the 2 techniques: It was found that the tail-cuff underestimated systolic and

diastolic blood pressure by 0.25±22.7 and 12.2±24.0 mmHg respectively. I have compared some 850 time-matched recording pairs and found that the measurements by the tail-cuff were on average 37.8±16.5 and 33.9±17.0 mmHg lower for systolic and diastolic blood pressure respectively, i.e. most of the differences in the readings found in my study were around the lower limit of agreement as determined by Feng et al. On further consideration of the study by *Feng et al*, (Feng et al., 2008) they did show a similar variability in the measurements seen in this study. They, however, more frequently observed central systolic blood pressure below 140mHg (approximately 40% of observed values) during tail-cuff protocol. They showed that the tail-cuff systematically overestimates central blood pressure when central blood pressure is below 140mmHg as measured simultaneously by telemetry. The difference between the two methods was judged to be approximately equally distributed around zero in their study.

I rarely observed central blood pressure below 130 mmHg during the tail-cuff protocol and the tail-cuff technique reproducibly underestimated the simultaneous recordings by the telemetry. Other researchers report very similar blood pressure (and heart rate) during the restraint in mice (Gross and Luft, 2003; Davern et al., 2014) and rats (Bidani et al., 1993; Xu et al., 1996; Swali et al., 2010).

Other studies that use the tail-cuff technique commonly report similar blood pressure values to those I obtained. For example, phenotypic screen of 37 strains of mice (Feng et al., 2009) using VPR tail-cuff system (same as in this study) reported 100±2 to 133±3 mmHg range of systolic blood pressures for those animals. Other laboratories had comparable results using the same (Familtseva et al., 2016) or different tail-cuff systems (Koutnikova et al., 2009; Hoile et al., 2015; Husain et al., 2015). Based on this, I believe that my results are valid for both telemetry and tailcuff recordings, and are not due to a systematic error with any of the techniques.

There are differences between the protocols and mouse strains used by *Feng et al* (2008), and the present study, which may have given rise to the differences in the results. Firstly, they show mixed data for C57Bl/6 and CD-1 male mice and higher ambient temperature, (Feng et al., 2008) whilst we used just C57Bl/6 mice. Secondly, they performed tail-cuff recordings in the room heated to 25-30°C. (Feng et al., 2008) Since we collected data during all stages of the tail-cuff protocol, including the acclimatisation period, we used pre-heated platforms and infra-red lamps during the recording sessions to maintain the temperature of the mouse's tail between 33-35°C. Therefore, there are several reasons why a difference in the results obtained by *Feng et al* (2008) and this study is observed.

6.5. An improved understanding of the tail cuff technique through simultaneous telemetry measuremen

I realised that the non-simultaneous telemetry blood pressure measurements, i.e. those taken before the animals were disturbed for the tail-cuff procedure, were similar to those obtained by the tail-cuff technique. Moreover, both telemetry (simultaneous and non-simultaneous recordings) and the tail-cuff, all reflected the expected hypertensive response that the pressor agent Ang II is associated with (Bodkin et al., 2014; Smillie et al., 2014) and the hypotensive response to such agents as CGRP (Aubdool et al., 2017).

Blood pressure is a complex phenomenon resulting from anatomical and physiological characteristics of the blood vessel that contain it and those of the circulatory network that it is part of. Therefore, it is fair to expect that blood pressure readings will differ between different locations within the arterial tree and these differences might not be the same among different species. Blood pressure changes from core to the periphery in humans are characterised by pressure amplification in the resistance vessels (London and Pannier, 2010; Nichols et al., 2011). Whereas in the mouse, based on experimental data and *in silico* modelling, there appears to be gradual dampening of the pressure towards the periphery (Aslanidou et al., 2016). The first evidence of this was published some 80 years ago when Byrom and Wilson (1938) reported that the pressure in the tail, as measured by the cuff method, is 10 mmHg lower than the pressure measured directly from the carotid artery. More recently it was shown that systolic blood pressure measurement from caudal artery in an anaesthetised rat is approximately 17 mmHg lower than the pressure measured directly from the femoral artery simultaneously (Wang et al., 2013). Although no such comparison was made in the mouse to my knowledge, the in silico model suggests that there is gradual decrease in blood pressure down the abdominal aorta in the mouse (Aslanidou et al., 2016), so the findings in the rat are also similarly true for the mouse.

The tail is an important thermoregulatory organ in rodents (Sittiracha et al., 1987; Cassell et al., 1988) that has both a rich blood supply and adrenergic innervation to enable efficient blood flow adjustment depending on external and internal parameters. It has been shown that the caudal artery receives between 1 - 5% of blood volume, depending on ambient temperature (Aslanidou et al., 2016). These and other characteristics of this vascular bed suggest that it is markedly different from that of the conducting vessels like aortic arch, where telemetric blood pressure measurements often take place in the mouse, including this study.

It is not entirely clear however if the difference between the simultaneous recordings is a consequence of the true difference in the two vascular beds that these two techniques take the

measurements from. Likewise, it is not clear to me if the similarity between the non-simultaneous recordings by the two techniques is due to the different changes in the tail artery and the central aorta during the stress. The check of the tail-cuff apparatus against mercury manometer suggested there is no calibration bias in the tail-cuff equipment, however I believe the test was limited and did not entirely exclude there may be a calibration bias.

The blood pressure wave contains far more information than the peak and trough values that nevertheless successfully guided the diagnosis in over hundred years. Before blood pressure could be quantitatively measured, pulse analysis was the centre of cardiovascular assessment, (see chapter 1). There are now new methods emerging that will allow quantitative analysis of the arterial waveforms (Nandi et al., 2018) that may lead to further insights into cardiovascular physiology. Telemetry allows continuous recording of central blood pressure wave at 1000Hz resolution in unrestrained conscious mice in home-cage environment. The most recent technology allows simultaneous measurement of the blood pressure wave, ECG, mouse activity and temperature in group-housed mice (Knot and Lee, 2016). This technology is necessary for the waveform analysis.

Telemetry, however, is more expensive to set up and run than the alternative non-invasive tail-cuff technique. New telemetry technology as described above is simply out of reach for many laboratories. Insertion of the telemetry probe requires major surgery and permanent occlusion of a major artery, typically left carotid, that was shown to affect blood flow to the brain in the short term (Polycarpou et al., 2016) and also induces acute changes to the vasculature (Tamaki et al., 2006). However, there are no studies to my knowledge that investigate any long term consequences of unilateral carotid artery occlusion. There is evidence that mice under 25 g body weight show signs of stress 28 days after the probe implantation as evidenced by non-recoverable body weight and increased sympathetic activation (Einstein et al., 2004). The weight and volume of the implantable telemetry probes (DSI, St.Paul USA) for small animals most commonly in use today range from 1.1 to 1.4 cm3 and 1.4 to 2.2 g. Thus, the probes alone count for between 5.6 and 8.8% of the body weight of a 25g mouse. The interaction of the telemetry probe on body weight is thus a significant limitation for studies involving female mice in particular, since the typical body weight for female mice at 16 weeks of age is below 21-22 g (Reed et al., 2007) for most commonly used strains, including the C57Bl/6J strain that claims over 43% of citations and is thus the most commonly used mouse strain in laboratories worldwide (Johnson, 2012).

Moreover, many disease models involve further surgical procedures and implantation of devices. The mouse angiotensin II – induced hypertension model involves subcutaneous implantation of a mini-pump to deliver angiotensin II. The typical dimensions of mini-pumps that are used in mice

are 1.5 cm length by 0.6 cm diameter with filled weight approximately 0.5 g. The combined weight of the pump and the telemetry device would typically account for 7.6 –10.8 % of weight for a 25g mouse. Insertion of the mini-pump is a surgical procedure that overall increases the severity of the procedure of telemetry-based studies. The length of telemetry studies may also be limited by the battery life. The probe can cause tissue damage that may manifest itself as the skin sores and may also limit the study duration. The cost and technical requirements associated with the telemetry equipment make it unfeasible for screening large numbers of animals.

6.6. Study limitations and future experiments

A primary limitation of this study is that we did not measure biomarkers of stress, such as the cortisol levels. We had discussed doing this, however it was difficult to work out how to do this without substantially increasing the numbers of mice used and possibly affecting blood pressure measurements. We instead relied on the more non-specific measure of blood pressure and heart rate to assess levels of stress, as this was the major measurement parameter within this study. I believe that it would be important to measure stress liability of the tail-cuff technique by way of measuring biomarkers of stress as the next step.

In chapter 3, a cross over study design was used to investigate the effect of the different handling techniques on central blood pressure. This potentially meant that I could not rule out interference between the handling techniques tested. This first study only included males. However, in later studies females were included to establish how they act in the protocol. The males and female mice responded in a remarkably similar manner in the whole tail cuff protocol. On the one hand, using both genders may have introduced more variability in the measurements. On the other hand including female mice added useful knowledge on the potential impact of gender and stress in the mice.

Since some studies lasted up to 3 months, this resulted in up to 13 weeks of age difference in the same mouse from start to finish of some experiments. Overall, no major drift in the cardiovascular responses were observed within these experiments. On some occasions, data from several experiments was combined, which meant that the actual age difference between the mice from different groups was up to 8 weeks. Although there is evidently a progressive blood pressure increase with age in mice (Barsha et al, 2016, Gros et al, 2002), the magnitude of this change until mice reach over one year of age is small and therefore the impact in the context of these experiments is not considered to be significant.

It is noted that activity records were taken and analysed in certain studies. This was useful information as it allowed a comparison with heart rate, on which activity impacts. Activity, or rather the physical effort whilst the mice were restrained could not be measured and therefore the impact of this physical effort on the blood pressure and heart rate increase during the tail-cuff protocol could not be assessed. This limitation can be addressed, for example, by collecting cortisol, a direct stress marker, following the tail-cuff protocol compared to following a period of high spontaneous activity in mice.

In this project CD1 and C57BL6/J mice were used. There was a striking difference in the ability to use these two species in that CD1 mice were very difficult to work with. The reason for this seemed to be very low blood flow in the tails of these mice despite using the same warming regiment as for the C57Bl6/J strain. This was one of the main reasons CD1 strain was not further used. All further studies were in C57/BL6 mice, which fits with the background strain that many genetically mice are raised and which many cardiovascular research labs use.

A further limitation is that the different studies involved different times of day. For the first study in chapter 3, all studies were in the morning. In chapter 4 and 5 the studies were more drawn out, meaning that sometimes the measurements were taken in the early afternoon. However, it is difficult to determine from the readings how this affected the overall results. Obviously it would have been better to have always carried out all the measurements at the same time of day, due to the additional variation that circadian rhythms may introduce.

In conclusion, relevant to hypothesis I hypothesised that I would be able to improve the handling technique in a manner that would substantially reduce stress associated with the tail cuff technique. This hypothesis was wrong as despite the well-developed handling research of Hurst and West, the cardiovascular responses to the three handling techniques that were tested in the context of the tail cuff protocol, were very similar. This research was achieved through the continuous measurement of mouse blood pressure by telemetry whilst carrying out all stages of the tail-cuff protocol. Additionally there was no influence in terms of the handler's gender, the habituation/training of them mice; despite previous publications. However, as a consequence of this project, I am able to recommend further adjustments and considerations for the tail-cuff protocol in addition to the manufacturer's recommendations.

- Habituate the animals to handling before commencing the experiments: Use the handling technique that you can reproduce most consistently and which, at the same time, causes least apparent distress in the mouse. The distress levels can be judged by mouse body posture, willingness of interaction etc.

- Training and Habituation: Although extensive training /habituating mice to the tail-cuff protocol did not offer significant advantage, more training sessions may be necessary for less experienced researchers.

- Temperature of the Room: Ambient temperature in the room where the tail-cuff protocol takes place should be no less than 25°C. Although the heat settings on the warming platform can be increased to level "4" to compensate for ambient temperature down to 22°C, higher heat settings may cause over-heating and in my experience were difficult to maintain the desired temperature range of 33-35°C for optimal vasodilation in the tail.

- Acclimatisation in the room: The mice should be acclimatised in the tail-cuff procedure room at least 30 minutes

- The Restraint Tube: Addition of a food pellet may encourage mice to walk into the tail-cuff holding tube more easily. Care should be taken to provide the adequate space in the restraint tube to avoid unnecessary discomfort, yet to minimise excessive movement. It is important to ensure that the mouse's nose can be seen in the nose cone to ensure that the mouse can breathe; nose movement can be monitored to monitor mice during the protocol.

- Acclimatisation in the Restraint Tube: Approximate acclimatisation time of 5 minutes in the restraint /tail-cuff holding tubes is often sufficient to achieve the required ambient temperature around the mouse.

- Tail-cuff Recording: Once the tail-cuff recording is initiated, the achieved volume increase of the tail should be no less than 15 µl and ideally 40 – 100 µl. Heating level on the platform can be increased (while monitoring the body surface temperature of the mouse) if insufficient tail swelling is observed.

- Total Restraint period: Optimal total restraint period for the mouse should not exceed 20 minutes. It was possible, however, to record mouse's blood pressure twice on the same day.

- To note – central blood pressure: It is important to remember that the tail-cuff technique does not provide the pressure measurements as seen in the aortic arch at the same time, the technique nevertheless is able to fairly reliably estimate the central blood pressure in "normotensive" mice and monitor the progression of hypertension in a manner that is proportional to the changes seen in the central circulation. Reliability of this estimate

depends on the many variables of the tail-cuff protocol, such as achieving the adequate temperature to ensure adequate vasodilation in the tail as one of the most important variables.

To conclude; this PhD project has allowed a series of studies to be performed that were designed to understand the limitations of the tail-cuff technique when compared with telemetry in studies involving the conscious mouse. The primary aim of minimising the stress associated with the tail-cuff procedure was difficult to achieve. However, the results in this thesis allow us to better understand the mouse blood pressure, in terms of central v peripheral blood pressure during the tail-cuff procedure is used and especially in terms of all the steps in the procedure as outlined above. To my knowledge this thesis has advanced as well as brought together and built on information from the few similar studies that have been carried out. It should be noted that at the time of writing (December 2019) the publication from this project (Wilde et al., 2017) has been cited 15 times (according to Scopus, Elsevier). This indicates the positive use that is being made of this project.

Bibliography

Agarwal, R. (2010). Regulation of circadian blood pressure: from mice to astronauts. Curr Opin Nephrol Hypertens *19*: 51–58.

Aird, W.C. (2011). Discovery of the cardiovascular system: from Galen to William Harvey. J. Thromb. Haemost. *9 Suppl 1*: 118–129.

Alawi, K.M., Aubdool, A.A., Liang, L., Wilde, E., Vepa, A., Psefteli, M.-P., et al. (2015). The sympathetic nervous system is controlled by transient receptor potential vanilloid 1 in the regulation of body temperature. FASEB J. *29*: 4285–4298.

Algire, G.H. (1949). Blood pressure measurements and changes in peripheral vascular bed of unanesthetized mice. FEDERATION PROCEEDINGS *8*: 349.

Aslanidou, L., Trachet, B., Reymond, P., Fraga-Silva, R.A., Segers, P., and Stergiopulos, N. (2016). A 1D model of the arterial circulation in mice. ALTEX *33*: 13–28.

Aubdool, A.A., Thakore, P., Argunhan, F., Smillie, S.-J., Schnelle, M., Srivastava, S., et al. (2017). A Novel αCalcitonin Gene-Related Peptide Analogue Protects Against End-Organ Damage in Experimental Hypertension, Cardiac Hypertrophy, and Heart Failure. Circulation *136*: 367–383.

Avolio, A.P., Butlin, M., and Walsh, A. (2010). Arterial blood pressure measurement and pulse wave analysis--their role in enhancing cardiovascular assessment. Physiol Meas *31*: R1–47.

Balcombe, J.P., Barnard, N.D., and Sandusky, C. (2004). Laboratory routines cause animal stress. Contemp. Top. Lab. Anim. Sci. *43*: 42–51.

Baldwin, F.M., Cook, H.B., and Nelson, V.E. (1924). Blood pressure in rats under deficient diets. American Journal of Physiology-Legacy Content *68*: 379–384.

Barnard, A.C., Hunt, W.A., Timlake, W.P., and Varley, E. (1966). A theory of fluid flow in compliant tubes. Biophys. J. *6*: 717–724.

Barr, J. (2014). Vascular medicine and surgery in ancient Egypt. J. Vasc. Surg. *60*: 260–263.

Barsha, G., Denton, K.M., and Mirabito Colafella, K.M. (2016). Sex- and age-related differences in arterial pressure and albuminuria in mice. Biol. Sex Differ. *7*: 57.

Bartels, K., Esper, S.A., and Thiele, R.H. (2016). Blood pressure monitoring for the anesthesiologist: A practical review. Anesth. Analg. *122*: 1866–1879.

Bassareo, P.P., and Crisafulli, A. (2019). Gender differences in hemodynamic regulation and cardiovascular adaptations to dynamic exercise. Curr Cardiol Rev.

Batchu, S.N., Smolock, E.M., Dyachenko, I.A., Murashev, A.N., and Korshunov, V.A. (2015). Autonomic dysfunction determines stress-induced cardiovascular and immune complications in mice. J. Am. Heart Assoc. *4*:

Bay, N.S.-Y., and Bay, B.-H. (2010). Greek anatomist herophilus: the father of anatomy. Anat. Cell Biol. *43*: 280–283.

Beale, A.L., Kaye, D.M., and Marques, F.Z. (2019). The role of the gut microbiome in sex differences in arterial pressure. Biol. Sex Differ. *10*: 22.

Bejan, A. (2005). The constructal law of organization in nature: tree-shaped flows and body size. J. Exp. Biol. *208*: 1677–1686.

Bejan, A., and Lorente, S. (2010). The constructal law of design and evolution in nature. Philos. Trans. R. Soc. Lond. B, Biol. Sci. *365*: 1335–1347.

Bejan, A., and Lorente, S. (2013). Constructal law of design and evolution: Physics, biology, technology, and society. J. Appl. Phys. *113*: 151301.

Belz, G.G. (1995). Elastic properties and Windkessel function of the human aorta. Cardiovasc Drugs Ther *9*: 73–83.

Bidani, A.K., Griffin, K.A., Picken, M., and Lansky, D.M. (1993). Continuous telemetric blood pressure monitoring and glomerular injury in the rat remnant kidney model. Am. J. Physiol. *265*: F391–8.

Bilton, K., and Zaslawski, C. (2016). Reliability of manual pulse diagnosis methods in traditional east asian medicine: A systematic narrative literature review. J. Altern. Complement. Med. *22*: 599–609.

Bland, J.M., and Altman, D.G. (2007). Agreement between methods of measurement with multiple observations per individual. J Biopharm Stat *17*: 571–582.

Bodkin, J.V., Thakore, P., Aubdool, A.A., Liang, L., Fernandes, E.S., Nandi, M., et al. (2014). Investigating the potential role of TRPA1 in locomotion and cardiovascular control during hypertension. Pharmacol. Res. Perspect. *2*: e00052.

Bonsmann, M.R. (1934). Blutdruckversuche an der Maus und Ratte mittels Photozelle. Archiv F. Experiment. Pathol. U. Pharmakol *176*: 460–467.

Booth, J. (1977). A short history of blood pressure measurement. Proc R Soc Med *70*: 793–799.

Brophy, G. (2009). The Sphygmograph: Pros-theses on the Body. Victorian Review *35*: 13–17.

Bruce Fye, W., and Willis Hurst, J. (1991). Carl Ludwig. Clin Cardiol *14*: 361–363.

Buñag, R.D. (1983). Facts and fallacies about measuring blood pressure in rats. Clin. Exp. Hypertens. A *5*: 1659–1681.

Buñag, R.D., and Butterfield, J. (1982). Tail-cuff blood pressure measurement without external preheating in awake rats. Hypertension *4*: 898–903.

Byrom, F.B., and Wilson, C. (1938). A plethysmographic method for measuring systolic blood pressure in the intact rat. J. Physiol. (Lond.) *93*: 301–304.

Cabanac, A., and Briese, E. (1992). Handling elevates the colonic temperature of mice. Physiol. Behav. *51*: 95–98.

Cameron, J.S., and Hicks, J. (1996). Frederick Akbar Mahomed and his role in the description of hypertension at Guy's Hospital. Kidney Int. *49*: 1488–1506.

Carey, R.M., Whelton, P.K., and 2017 ACC/AHA Hypertension Guideline Writing Committee (2018). Prevention, detection, evaluation, and management of high blood pressure in adults: synopsis of the 2017 american college of cardiology/american heart association hypertension guideline. Ann. Intern. Med. *168*: 351–358.

Cassell, J.F., McLachlan, E.M., and Sittiracha, T. (1988). The effect of temperature on neuromuscular transmission in the main caudal artery of the rat. J. Physiol. (Lond.) *397*: 31–49.

Chen, Y., and Yu, T. (2018). Testosterone mediates hyperthermic response of mice to heat exposure. Life Sci. *214*: 34–40.

Chowienczyk, P. (2011). Pulse wave analysis: what do the numbers mean? Hypertension *57*: 1051–1052.

Chruscinski, A., Brede, M.E., Meinel, L., Lohse, M.J., Kobilka, B.K., and Hein, L. (2001). Differential distribution of β-adrenergic receptor subtypes in blood vessels of knockout mice lacking β1-or β2adrenergic receptors. Molecular Pharmacology *60*: 955–962.

Daugherty, A., Rateri, D., Hong, L., and Balakrishnan, A. (2009). Measuring blood pressure in mice using volume pressure recording, a tail-cuff method. J. Vis. Exp.

Davern, P.J., Chowdhury, S., Jackson, K.L., Nguyen-Huu, T.-P., and Head, G.A. (2014). GABAA receptor dysfunction contributes to high blood pressure and exaggerated response to stress in Schlager genetically hypertensive mice. J. Hypertens. *32*: 352–362.

Diaz, J.T., and Levy, S.E. (1939). An indirect method for repeated determinations of blood pressure of rats. Proceedings of the Society for Experimental Biology and Medicine *40*: 402–407.

Drüeke, T.B., and Devuyst, O. (2019). Blood pressure measurement in mice: tail-cuff or telemetry? Kidneyinternational-online.org *96*: 36.

Durant, R.R. (1927). Blood pressure in the rat. American Journal of Physiology-Legacy Content *81*: 679–685.

Einstein, R., Billing, R.L., Singh, A., and Chin, I. (2004). Implanted telemetry transmitters alter the noradrenergic response in vas deferens from mice. Altern. Lab. Anim. *32 Suppl 1A*: 171–176.

Familtseva, A., Chaturvedi, P., Kalani, A., Jeremic, N., Metreveli, N., Kunkel, G.H., et al. (2016). Toll-like receptor 4 mutation suppresses hyperhomocysteinemia- induced hypertension. Am. J. Physiol. Cell Physiol. *311*: ajpcell.00088.2016.

Feng, M., Deerhake, M.E., Keating, R., Thaisz, J., Xu, L., Tsaih, S.-W., et al. (2009). Genetic analysis of blood pressure in 8 mouse intercross populations. Hypertension *54*: 802–809.

Feng, M., Whitesall, S., Zhang, Y., Beibel, M., D'Alecy, L., and DiPetrillo, K. (2008). Validation of volume-pressure recording tail-cuff blood pressure measurements. Am. J. Hypertens. *21*: 1288–1291.

Fishel SR, Muth ER, Hoover AW. Establishing appropriate physiological baseline procedures for real-time physiological measurement. Journal of Cognitive Engineering and Decision Making, Volume 1, Number 3, 2007, pp. 286–308. DOI 10.1518/155534307X255636. 2007

Foster, B. (1866). The sphygmograph in English medical practice. Lancet *87*: 634.

Friedman, M., and Freed, S.C. (1949). Microphonic manometer for indirect determination of systolic blood pressure in the rat. Proc Soc Exp Biol Med *70*: 670–672.

Geddes, L.A. (2002). The first accurate measurement of systolic and diastolic blood pressure. IEEE Eng Med Biol Mag *21*: 102–103.

Geddes, L.A. (2013). Handbook of blood pressure measurement (Springer Science & Business Media).

Ghasemzadeh, N., and Zafari, A.M. (2011). A brief journey into the history of the arterial pulse. Cardiol Res Pract *2011*: 164832.

Gouveia, K., and Hurst, J.L. (2013). Reducing mouse anxiety during handling: effect of experience with handling tunnels. PLoS One *8*: e66401.

Gouveia, K., and Hurst, J.L. (2017). Optimising reliability of mouse performance in behavioural testing: the major role of non-aversive handling. Sci. Rep. *7*: 44999.

Griffith, J.Q. (1934). Indirect method for determining blood pressure in small animals. Exp. Biol. Med. *32*: 394–396.

Griffith, J.Q., and Collins, L.H. (1933). A method for obtaining blood pressure by arterial compression and simulataneous capillary observation. Am. Heart J. *8*: 671–675.

Gross, V., and Luft, F.C. (2003). Exercising restraint in measuring blood pressure in conscious mice. Hypertension *41*: 879–881.

Grundfest, H., Hay, J.J., and Feitelberg, S. (1945). A strain gage recorder for physiological volume, pressure and deformation measurements. Science *101*: 255–256.

Haggerty, C.M., Mattingly, A.C., Gong, M.C., Su, W., Daugherty, A., and Fornwalt, B.K. (2015). Telemetric Blood Pressure Assessment in Angiotensin II-Infused ApoE-/- Mice: 28 Day Natural History and Comparison to Tail-Cuff Measurements. PLoS One *10*: e0130723.

Hales, S. (1732). Statistical Essays: Containing Haemastatics; or an Account of Some Hydraulic and Hydrostatical Experiments.

Hales, S. (1733). Statical Essays: containing haemostaticks; or An Account of some Hydraulick and Hydrostatical Experiments made on the blood and Blood-Vessels of Animals (New York: Hafner Publishing Company).

Hametner, B., and Wassertheurer, S. (2017). Pulse waveform analysis: is it ready for prime time? Curr Hypertens Rep *19*: 73.

Hamilton, S. (2016). On the Cruelty to Animals Act, 15 August 1876. BRANCH: Britain, Representation and Nineteenth-Century History.

Hamilton, W.F. (1936). Physiologic relationships between intrathoracic, intraspinal and arterial pressures. JAMA *107*: 853.

Hamilton, W.F., Brewer, G., and Brotman, I. (1934). Pressure pulse contours in the intact animal. American Journal of Physiology-Legacy Content *107*: 427–435.

Harre, R. (1983). Stephen Hales: The Circulation of Sap in Plants. In Great Scientific Experiments: Twenty Experiments That Changed Our View of the World, (Oxford University Press), pp 52–58.

Hay, J. (1931). A British medical association lecture on THE SIGNIFICANCE OF A RAISED BLOOD PRESSURE. Br. Med. J. *2*: 43–47.

Hedrich, H. (2004). The laboratory mouse (Academic Press).

Henig, R.M. (2017). The monk in the garden: the lost and found genius of Gregor Mendel, the father of genetics (Houghton Mifflin Harcourt).

Henry, J.P., Meehan, J.P., Stephens, P., and Santisteban, G.A. (1965). Arterial pressure in CBA mice as related to age. J Gerontol *20*: 239–243.

Henry, J.P., Meehan, J.P., and Stephens, P.M. (1967). The use of psychosocial stimuli to induce prolonged systolic hypertension in mice. Psychosom. Med. *29*: 408–432.

Herisson, J. (1835). The Sphygmometer, an Instrument that Makes the Action of the Arteries Apparant to the Eye (Longman, Rees, Orme and Brown, and Longman).

Hill, L., and Barnard, H. (1897). A simple and accurate form of sphygmometer or arterial pressure gauge contrived for clinical use. Br. Med. J. *2*: 904.

Hoile, S.P., Grenfell, L.R., Hanson, M.A., Lillycrop, K.A., and Burdge, G.C. (2015). Fat and carbohydrate intake over three generations modify growth, metabolism and cardiovascular phenotype in female mice in an age-related manner. PLoS One *10*: e0134664.

Home Office Annual Statistics of Scientific Procedures on Living Animals Great Britain 2016.

Home Office Annual Statistics of Scientific Procedures on Living Animals, Great Britain 2018.

Hurst, J.L., and West, R.S. (2010). Taming anxiety in laboratory mice. Nat. Methods *7*: 825–826.

Husain, K., Suarez, E., Isidro, A., Hernandez, W., and Ferder, L. (2015). Effect of paricalcitol and enalapril on renal inflammation/oxidative stress in atherosclerosis. World J Biol Chem *6*: 240–248.

Huxley, V.H., and Kemp, S.S. (2018). Sex-Specific Characteristics of the Microcirculation. Adv. Exp. Med. Biol. *1065*: 307–328.

Irvine, R.J., White, J., and Chan, R. (1997). The influence of restraint on blood pressure in the rat. J. Pharmacol. Toxicol. Methods *38*: 157–162.

Jennings JR, Kamarck T, Stewart C, Eddy M. Alternate cardiovascular baseline assessment techniques: Vanilla or resting baseline. 1992

Ji, H., Zheng, W., Li, X., Liu, J., Wu, X., Zhang, M.A., et al. (2014). Sex-specific T-cell regulation of angiotensin II-dependent hypertension. Hypertension *64*: 573–582.

Johns, C., Gavras, I., Handy, D.E., Salomao, A., and Gavras, H. (1996). Models of experimental hypertension in mice. Hypertension *28*: 1064–1069.

Johnson, E.O., Kamilaris, T.C., Chrousos, G.P., and Gold, P.W. (1992). Mechanisms of stress: a dynamic overview of hormonal and behavioral homeostasis. Neurosci. Biobehav. Rev. *16*: 115–130.

Johnson, M. (2012). Laboratory mice and rats. MATER. METHODS *2*:

Kahn, M.H. (1924). A method for recording continuous blood pressure. Exp. Biol. Med. *22*: 166–168.

Kanad, S. (1891). Science of Sphygmica or Sage Kanad on Pulse, An English Translation with Sansskrit Passages (Calcutta: S.C.Addy).

Kannel, W.B. (2000). Elevated systolic blood pressure as a cardiovascular risk factor. Am. J. Cardiol. *85*: 251–255.

Kaptchuk, T.J. (1983). The Four Examinations: Signs and Symptoms. In Chinese Medicine: The Web That Has No Weaver, (Rider), pp 138–177.

Keeney, A.J., Hogg, S., and Marsden, C.A. (2001a). Alterations in core body temperature, locomotor activity, and corticosterone following acute and repeated social defeat of male NMRI mice. Physiol. Behav. *74*: 177–184.

Keeney, A.J., Hogg, S., and Marsden, C.A. (2001b). Alterations in core body temperature, locomotor activity, and corticosterone following acute and repeated social defeat of male NMRI mice. Physiol. Behav. *74*: 177–184.

Kilkenny, C., Browne, W.J., Cuthill, I.C., Emerson, M., and Altman, D.G. (2010). Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research. PLoS Biol. *8*: e1000412.

Knot, H.J., and Lee, D. (2016). A Novel Freely Moving Animal Based Blood Pressure, ECG, and Dual Body Temperature Telemetry System for Group Housed Mice in Social Context. FASEB J. *30*:

Kolls, A.C. (1920). Continuous Blood Pressure Tracingss in Man: an Apparatus. J. Pharmacol. Exp. Ther. *15*: 433–441.

Kotchen, T.A. (2011). Historical trends and milestones in hypertension research: a model of the process of translational research. Hypertension *58*: 522–538.

Koutnikova, H., Laakso, M., Lu, L., Combe, R., Paananen, J., Kuulasmaa, T., et al. (2009). Identification of the UBP1 locus as a critical blood pressure determinant using a combination of mouse and human genetics. PLoS Genet. *5*: e1000591.

Kramer, K., van de Weerd, H., Mulder, A., Heijningen, C. Van, Baumans, V., Remie, R., et al. (2004). Effect of conditioning on the increase of heart rate and body temperature provoked by handling in the mouse. Altern. Lab. Anim. *32 Suppl 1A*: 177–181.

Kramer, K., Voss, H.P., Grimbergen, J.A., Mills, P.A., Huetteman, D., Zwiers, L., et al. (2000). Telemetric monitoring of blood pressure in freely moving mice: a preliminary study. Lab Anim *34*: 272–280.

Kratz, M.T., Schirmer, S.H., Baumhäkel, M., and Böhm, M. (2016). Improvement of endothelial function in a murine model of mild cholesterol-induced atherosclerosis by mineralocorticoid antagonism. Atherosclerosis *251*: 291–298.

Krege, J.H., Hodgin, J.B., Hagaman, J.R., and Smithies, O. (1995). A noninvasive computerized tail-cuff system for measuring blood pressure in mice. Hypertension *25*: 1111–1115.

Kurande, V., Waagepetersen, R., Toft, E., Prasad, R., and Raturi, L. (2012). Repeatability of pulse diagnosis and body constitution diagnosis in traditional indian ayurveda medicine. Glob. Adv. Health Med. *1*: 36– 42.

Kurtz, T.W., Griffin, K.A., Bidani, A.K., Davisson, R.L., Hall, J.E., and Subcommittee of Professional and Public Education of the American Heart Association (2005a). Recommendations for blood pressure measurement in humans and experimental animals. Part 2: Blood pressure measurement in experimental animals: a statement for professionals from the subcommittee of professional and public education of the American Heart Association council on high blood pressure research. Hypertension *45*: 299–310.

Kurtz, T.W., Griffin, K.A., Bidani, A.K., Davisson, R.L., Hall, J.E., and Subcommittee of Professional and Public Education of the American Heart Association Council on High Blood Pressure Research (2005b). Recommendations for blood pressure measurement in humans and experimental animals: part 2: blood pressure measurement in experimental animals: a statement for professionals from the Subcommittee of Professional and Public Education of the American Heart Association Council on High Blood Pressure Research. Arterioscler. Thromb. Vasc. Biol. *25*: e22–33.

Kvetnanský, R., and Sabban, E.L. (1998). Stress and molecular biology of neurotransmitter-related enzymes. Ann. N. Y. Acad. Sci. *851*: 342–356.

Lambert, E.H., and Wood, E.H. (1947). The use of a resistance wire, strain gauge manometer to measure intraarterial pressure. Proc Soc Exp Biol Med *64*: 186–190.

Li, P., Sur, S.H., Mistlberger, R.E., and Morris, M. (1999). Circadian blood pressure and heart rate rhythms in mice. Am. J. Physiol. *276*: R500–4.

Lilly, J.C. (1942). The electrical capacitance diaphragm manometer. Review of Scientific Instruments *13*: 34–37.

Lim, S.S., Vos, T., Flaxman, A.D., Danaei, G., Shibuya, K., Adair-Rohani, H., et al. (2012). A comparative risk assessment of burden of disease and injury attributable to 67 risk factors and risk factor clusters in 21 regions, 1990-2010: a systematic analysis for the Global Burden of Disease Study 2010. Lancet *380*: 2224–2260.

Lindenstrøm, E., Boysen, G., and Nyboe, J. (1995). Influence of systolic and diastolic blood pressure on stroke risk: a prospective observational study. Am. J. Epidemiol. *142*: 1279–1290.

Lloyd, G.E.R. (1979). The Development of Empirical Research. In Magic, Reason and Experience: Studies in the Origin and Development of Greek Science, (Cambridge University Press), pp 126 – 169.

London, G.M., and Pannier, B. (2010). Arterial functions: how to interpret the complex physiology. Nephrol. Dial. Transplant. *25*: 3815–3823.

Luther, J.M., and Fogo, A.B. (2019). Under pressure—how to assess blood pressure in rodents: tail-cuff? Kidneyinternational-online.org *96*: 34–35.

Mach, E. (2016). On the theory of the sphygmograph. Sci Context *29*: 435–440.

Malkoff, J. (2005). Non-invasive blood pressure for mice and rats. Animal Lab News, Kent Scientific Corporation 1–12.

Maranon, R., and Reckelhoff, J.F. (2013). Sex and gender differences in control of blood pressure. Clin. Sci. *125*: 311–318.

Marshall, N.J., Liang, L., Bodkin, J., Dessapt-Baradez, C., Nandi, M., Collot-Teixeira, S., et al. (2013). A role for TRPV1 in influencing the onset of cardiovascular disease in obesity. Hypertension *61*: 246–252.

McMaster, P.D. (1941). A method to determine the peripheral arterial blood pressure in the mouse. J. Exp. Med. *74*: 29–40.

Meer, C. Van der, and Bekkum, D.W. Van (1959). The mechanism of radiation protection by histamine and other biological amines. International Journal of Radiation Biology and Related Studies in Physics, Chemistry and Medicine *1*: 5–23.

Mills, P.A., Huetteman, D.A., Brockway, B.P., Zwiers, L.M., Gelsema, A.J., Schwartz, R.S., et al. (2000). A new method for measurement of blood pressure, heart rate, and activity in the mouse by radiotelemetry. J. Appl. Physiol. *88*: 1537–1544.

Mishra, L., Singh, B.B., and Dagenais, S. (2001). Ayurveda: a historical perspective and principles of the traditional healthcare system in India. Altern Ther Health Med *7*: 36–42.

Moberg, G.P., and Mench, J.A. (2000). The biology of animal stress: basic principles and implications for animal welfare (books.google.com).

Moore, N. (1897). A Lecture on the Pulse. Delivered at St Bartholomew's Hospital on Nov. 11th, 1897, preliminary to the Course of Lectures on Medicine. The Lanet.

Moser, M. (1986). Historical perspective on the management of hypertension. Am. J. Med. *80*: 1–11.

Mousa, T.M., Schiller, A.M., and Zucker, I.H. (2014). Disruption of cardiovascular circadian rhythms in mice post myocardial infarction: relationship with central angiotensin II receptor expression. Physiol. Rep. *2*:

Mukaka, M.M. (2012). Statistics corner: A guide to appropriate use of correlation coefficient in medical research. Malawi Med. J. *24*: 69–71.

Myers, M.G. (2014). Replacing manual sphygmomanometers with automated blood pressure measurement in routine clinical practice. Clin. Exp. Pharmacol. Physiol. *41*: 46–53.

Nandi, M., Venton, J., and Aston, P.J. (2018). A novel method to quantify arterial pulse waveform morphology: attractor reconstruction for physiologists and clinicians. Physiological Measurement.

Naqvi, N.H., and Blaufox, M.D. (1998). Blood Pressure Measurement: An Illustrated History (Parthenon Publishing).

Nelson, M.R., Stepanek, J., Cevette, M., Covalciuc, M., Hurst, R.T., and Tajik, A.J. (2010). Noninvasive measurement of central vascular pressures with arterial tonometry: clinical revival of the pulse pressure waveform? Mayo Clin. Proc. *85*: 460–472.

Nichols, W., O'Rourke, M., and Charalambos, V. (2011). Central Arterial Pressure. In McDonald's Blood

Flow in Arteries, Sixth Edition : Theoretical, Experimental and Clinical Principles, (CRC Press), pp 569–578.

O'Brien, E., and Fitzgerald, D. (1994). The history of blood pressure measurement. J Hum Hypertens *8*: 73–84.

O'Brien, K.A., Birch, S., Abbas, E., Movsessian, P., Hook, M., and Komesaroff, P.A. (2013). Traditional East Asian medical pulse diagnosis: a preliminary physiologic investigation. J. Altern. Complement. Med. *19*: 793–798.

O'Rourke, M.F., and Seward, J.B. (2006). Central arterial pressure and arterial pressure pulse: new views entering the second century after Korotkov. Mayo Clin. Proc. *81*: 1057–1068.

Oparil, S. (2016). Sy 11-3 hypertension in women: more dangerous than in men? J. Hypertens. *34 Suppl 1*:

e366.

Osler, W. (1912). High blood pressure: its associations, advantages, and disadvantages. BMJ *2*: 1173–1177.

Patwardhan, K. (2012). The history of the discovery of blood circulation: unrecognized contributions of Ayurveda masters. Adv Physiol Educ *36*: 77–82.

Pickering, T.G., Hall, J.E., Appel, L.J., Falkner, B.E., Graves, J., Hill, M.N., et al. (2005). Recommendations for blood pressure measurement in humans and experimental animals: part 1: blood pressure measurement in humans: a statement for professionals from the Subcommittee of Professional and Public Education of the American Heart Association Council on High Blood Pressure Research. Circulation *111*: 697–716.

Polycarpou, A., Hricisák, L., Iring, A., Safar, D., Ruisanchez, É., Horváth, B., et al. (2016). Adaptation of the Cerebrocortical Circulation to Carotid Artery Occlusion Involves Blood Flow Redistribution between Cortical Regions and is Independent of eNOS. Am. J. Physiol. Heart Circ. Physiol. *311*: ajpheart.00197.2016.

Rampling, M.W. (2007). History of Hemorheology. In Handbook of Hemorheology and Hemodynamics, O.K. Baskurt, M.R. Hardeman, M.W. Rampling, and H.J. Meiselman, eds. (IOS press), pp 3–17.

Razavi, M.S., Shirani, E., Salimpour, M.R., and Kassab, G.S. (2014). Constructal law of vascular trees for facilitation of flow. PLoS One *9*: e116260.

Reckelhoff, J.F. (2001). Gender differences in the regulation of blood pressure. Hypertension *37*: 1199–1208.

Reckelhoff, J.F. (2018). Sex differences in regulation of blood pressure. Adv. Exp. Med. Biol. *1065*: 139– 151.

Reckelhoff, J.F., Romero, D.G., and Yanes Cardozo, L.L. (2019). Sex, oxidative stress, and hypertension: insights from animal models. Physiology (Bethesda) *34*: 178–188.

Reddy, A.K., Taffet, G.E., Madala, S., Michael, L.H., Entman, M.L., and Hartley, C.J. (2003). Noninvasive blood pressure measurement in mice using pulsed Doppler ultrasound. Ultrasound Med Biol *29*: 379–385.

Reed, D.R., Bachmanov, A.A., and Tordoff, M.G. (2007). Forty mouse strain survey of body composition. Physiol. Behav. *91*: 593–600.

Reynolds, R.P., Kinard, W.L., Degraff, J.J., Leverage, N., and Norton, J.N. (2010). Noise in a laboratory animal facility from the human and mouse perspectives. J. Am. Assoc. Lab. Anim. Sci. *49*: 592–597.

Rogers, G., and Oosthuyse, T. (2000). A comparison of the indirect estimate of mean arterial pressure calculated by the conventional equation and calculated to compensate for a change in heart rate. International Journal of Sports Medicine *21*: 90–95.

Russell, W.M., Burch, R.L., and Hume, C.W. (1959). The principles of humane experimental technique.

Saba, M.M., Ventura, H.O., Saleh, M., and Mehra, M.R. (2006). Ancient Egyptian medicine and the concept of heart failure. J Card Fail *12*: 416–421.

Safar, M.E., and Jankowski, P. (2009). Central blood pressure and hypertension: role in cardiovascular risk assessment. Clin. Sci. *116*: 273–282.

Saklayen, M.G., and Deshpande, N.V. (2016). Timeline of history of hypertension treatment. Front. Cardiovasc. Med. *3*: 3.

Sanchez-Alavez, M., Alboni, S., and Conti, B. (2011). Sex- and age-specific differences in core body temperature of C57Bl/6 mice. Age (Omaha) *33*: 89–99.

Sarazan, R.D., and Schweitz, K.T.R. (2009). Standing on the shoulders of giants: Dean Franklin and his remarkable contributions to physiological measurements in animals. Adv Physiol Educ *33*: 144–156.

Savas, Ü., Wei, S., Hsu, M.-H., Falck, J.R., Guengerich, F.P., Capdevila, J.H., et al. (2016). 20Hydroxyeicosatetraenoic Acid (HETE)-dependent Hypertension in Human Cytochrome P450 (CYP) 4A11 Transgenic Mice: Normalization of blood pressure by sodium restriction, hydroclrothiazide, or blockade of the type 1 angiotensin II receptor. J. Biol. Chem. *291*: 16904–16919.

Sayk, F., Wobbe, I., Twesten, C., Meusel, M., Wellhöner, P., Derad, I., et al. (2015). Prolonged blood pressure elevation following continuous infusion of angiotensin II-a baroreflex study in healthy humans. Am. J. Physiol. Regul. Integr. Comp. Physiol. *309*: R1406–14.

Scotland, R.S., Madhani, M., Chauhan, S., Moncada, S., Andresen, J., Nilsson, H., et al. (2005). Investigation of vascular responses in endothelial nitric oxide synthase/cyclooxygenase-1 double-knockout mice: key role for endothelium-derived hyperpolarizing factor in the regulation of blood pressure in vivo. Circulation *111*: 796–803.

Segadal, L., and Matre, K. (1987). Blood velocity distribution in the human ascending aorta. Circulation *76*: 90–100.

Sesso, H.D., Stampfer, M.J., Rosner, B., Hennekens, C.H., Gaziano, J.M., Manson, J.E., et al. (2000). Systolic and diastolic blood pressure, pulse pressure, and mean arterial pressure as predictors of cardiovascular disease risk in Men. Hypertension *36*: 801–807.

Shadwick, R.E. (1999). Mechanical design in arteries. J. Exp. Biol. *202*: 3305–3313.

Sheward, W.J., Naylor, E., Knowles-Barley, S., Armstrong, J.D., Brooker, G.A., Seckl, J.R., et al. (2010). Circadian control of mouse heart rate and blood pressure by the suprachiasmatic nuclei: behavioral effects are more significant than direct outputs. PLoS One *5*: e9783.

Shu-he, W. (2007). The Pulse Classic: A Translation of the Mai Jing by Wang Shu-he (Blue Poppy Press).

Shuler, R.H., Kupperman, H.S., and Hamilton, W.F. (1944). Comparison of direct and indirect blood pressure measurements in rats. American Journal of Physiology-Legacy Content *141*: 625–629.

Sikora, M., Konopelski, P., Pham, K., Wyczalkowska-Tomasik, A., and Ufnal, M. (2016). Repeated restraint stress produces acute and chronic changes in hemodynamic parameters in rats. Stress *19*: 621–629.

Sittiracha, T., McLachlan, E.M., and Bell, C. (1987). The innervation of the caudal artery of the rat. Neuroscience *21*: 647–659.

Smillie, S.-J., King, R., Kodji, X., Outzen, E., Pozsgai, G., Fernandes, E., et al. (2014). An ongoing role of αcalcitonin gene-related peptide as part of a protective network against hypertension, vascular hypertrophy, and oxidative stress. Hypertension *63*: 1056–1062.

Smith, C.R., and Bickley, W.H. (1964). The measurement of blood pressure in the human body. The Measurement of Blood Pressure in the Human Body *5006*:

Sorge, R.E., Martin, L.J., Isbester, K.A., Sotocinal, S.G., Rosen, S., Tuttle, A.H., et al. (2014). Olfactory exposure to males, including men, causes stress and related analgesia in rodents. Nat. Methods *11*: 629–632.

Still, J.W., and Whitcomb, E.R. (1956). Technique for permanent long-term intubation of rat aorta. J. Lab. Clin. Med. *48*: 152–154.

Stuckey, G.C. (2004). Application of physical principles in the development of tonometry. Clin Experiment Ophthalmol *32*: 633–636.

Swali, A., McMullen, S., and Langley-Evans, S.C. (2010). Prenatal protein restriction leads to a disparity between aortic and peripheral blood pressure in Wistar male offspring. J. Physiol. (Lond.) *588*: 3809–3818.

Swoap, S.J., Li, C., Wess, J., Parsons, A.D., Williams, T.D., and Overton, J.M. (2008). Vagal tone dominates autonomic control of mouse heart rate at thermoneutrality. Am. J. Physiol. Heart Circ. Physiol. *294*: H1581–8.

Swoap, S.J., Overton, J.M., and Garber, G. (2004). Effect of ambient temperature on cardiovascular parameters in rats and mice: a comparative approach. Am. J. Physiol. Regul. Integr. Comp. Physiol. *287*: R391–6.

Syme, C., Abrahamowicz, M., Leonard, G.T., Perron, M., Richer, L., Veillette, S., et al. (2009). Sex differences in blood pressure and its relationship to body composition and metabolism in adolescence. Arch. Pediatr. Adolesc. Med. *163*: 818–825.

Tamaki, M., Kidoguchi, K., Mizobe, T., Koyama, J., Kondoh, T., Sakurai, T., et al. (2006). Carotid artery occlusion and collateral circulation in C57Black/6J mice detected by synchrotron radiation microangiography. Kobe J Med Sci *52*: 111–118.

Thomas, G.D. (2011). Neural control of the circulation. Adv Physiol Educ *35*: 28–32.

Timmermann, C. (2006). Hexamethonium, Hypertension and Pharmaceutical Innovation: The Transformation of an Experimental Drug in Post-war Britain. In Devices and Designs: Medical Technologies in Historical Perspective, C. Timmermann, and J. Anderson, eds. (Palgrave Macmillan), p 171.

Tokizawa, K., Yoda, T., Uchida, Y., Kanosue, K., and Nagashima, K. (2015). Estimation of the core temperature control during ambient temperature changes and the influence of circadian rhythm and metabolic conditions in mice. J Therm Biol *51*: 47–54.

Treacher, D. The Heart in Fink, M., Hayes, M. and Soni, N. (2008). Classic Papers in Critical Care. 2nd ed. Springer, pp.59-86.

Ulrich-Lai, Y.M., and Herman, J.P. (2009). Neural regulation of endocrine and autonomic stress responses. Nat. Rev. Neurosci. *10*: 397–409.

van Nimwegen, C., van Eijnsbergen, B., Boter, J., and Mullink, J.W. (1973). A simple device for indirect measurement of blood pressure in mice. Lab Anim *7*: 73–84.

Vlachopoulos, C., O'Rourke, M., and Nichols, W.W. (2011). Introduction in McDonald's blood flow in arteries: theoretical, experimental and clinical principles (CRC press).

Wallis, F. (2000). Signs and senses: diagnosis and prognosis in early medieval pulse and urine texts. Soc. Hist. Med. *13*: 265–278.

Wang, H., and Cheng, Y. (2005). A quantitative system for pulse diagnosis in Traditional Chinese Medicine. Conf. Proc. IEEE Eng. Med. Biol. Soc. *6*: 5676–5679.

Wang, L., Wang, X., Qu, H.Y., Jiang, S., Zhang, J., Fu, L., et al. (2017). Role of Kidneys in Sex Differences in Angiotensin II-Induced Hypertension. Hypertension *70*: 1219–1227.

Wang, S., Luo, M., Zhang, Z., Gu, J., Chen, J., Payne, K.M., et al. (2016). Zinc deficiency exacerbates while zinc supplement attenuates cardiac hypertrophy in high-fat diet-induced obese mice through modulating p38 MAPK-dependent signaling. Toxicol. Lett. *258*: 134–146.

Wang, Y., Cong, Y., Li, J., Li, X., Li, B., and Qi, S. (2013). Comparison of invasive blood pressure measurements from the caudal ventral artery and the femoral artery in male adult SD and Wistar rats. PLoS One *8*: e60625.

Weinman, J., Bicher, C., and Levy, D. (1960). Application of a photoconductive cell to the study of peripheral circulation in limbs of animals and man. J. Appl. Physiol. *15*: 317–320.

Whelton, P.K., Carey, R.M., Aronow, W.S., Casey, D.E., Collins, K.J., Dennison Himmelfarb, C., et al. (2017). 2017 ACC/AHA/AAPA/ABC/ACPM/AGS/APHA/ASH/ASPC/NMA/PCNA guideline for the prevention, detection, evaluation, and management of high blood pressure in adults: A report of the American College of Cardiology/American Heart Association task force on clinical practice guidelines. J. Am. Coll. Cardiol. *71*: 2199–2269.

Whitesall, S.E., Hoff, J.B., Vollmer, A.P., and D'Alecy, L.G. (2004). Comparison of simultaneous measurement of mouse systolic arterial blood pressure by radiotelemetry and tail-cuff methods. Am. J. Physiol. Heart Circ. Physiol. *286*: H2408–15.

Wilde E, Aubdool AA, Thakore P, Baldissera L, Alawi KM, Keeble J, et al. Tail-Cuff Technique and Its Influence on Central Blood Pressure in the Mouse. J Am Heart Assoc. 2017 Jun 27;6(6).

Williams, B., Lacy, P.S., Thom, S.M., Cruickshank, K., Stanton, A., Collier, D., et al. (2006). Differential impact of blood pressure-lowering drugs on central aortic pressure and clinical outcomes: principal results of the Conduit Artery Function Evaluation (CAFE) study. Circulation *113*: 1213–1225.

Williams, J.R., Harrison, T.R., and Grollman, A. (1939). A simple method for determining the systolic blood pressure of the unanesthetized rat. J. Clin. Invest. *18*: 373–376.

Williams, T.D., Chambers, J.B., Henderson, R.P., Rashotte, M.E., and Overton, J.M. (2002). Cardiovascular responses to caloric restriction and thermoneutrality in C57BL/6J mice. Am. J. Physiol. Regul. Integr. Comp. Physiol. *282*: R1459–67.

Woodbury, R.A., and Hamilton, W.F. (1937). Blood pressure studies in small animals. American Journal of Physiology-Legacy Content *119*: 663–674.

Wu, H.C., and Visscher, M.B. (1948). Adaptation of the tail plethysmograph to blood pressure measurement in the mouse with some observations on the effects of temperature. American Journal of Physiology-Legacy Content *153*: 330–335.

Xu, Q., Liu, Y., Gorospe, M., Udelsman, R., and Holbrook, N.J. (1996). Acute hypertension activates mitogen-activated protein kinases in arterial wall. J. Clin. Invest. *97*: 508–514.